GENE EXPRESSION ANALYSIS IN MEASLES VIRUS RESEARCH

by

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Abstract

The sequencing of the human genome ushered in the era of high-throughput biology. Rapid, whole cell analysis is replacing the molecular reductionist approach of the last century. Microarrays are one of the main high-throughput technologies in this field, allowing researchers to query the whole transcriptome of the cell in one experiment. Learning to harness the power of this technology is a significant research problem that requires expertise in biology, statistics and computation. Towards that goal, this work examines three projects exploring the role of microarray analysis in biological research: an *in vitro* infection model, acute disease in humans and vaccination in mice.

To test the technology in a highly controlled system, an *in vitro* infection model was developed where monocyte-derived dendritic cells were infected with measles virus and RNA was extracted over 24 hours. There were 1553 significantly regulated genes during this time, with nearly 60% of them down regulated. The results were compared to other *in vitro* infection systems, which highlighted a group of genes that formed a core response to all the pathogens, including 2'5' oligoadenylate synthetase, Mx and interferon response factors 1 and 7. The analysis also showed that measles virus is the only pathogen that does not induce dsRNA-dependent protein kinase above its constitutive expression level. Measles also induced a robust interferon-α response in contrast to the other pathogens. These results showed that microarray analysis could provide a modular view of the immune response.

Since microarrays worked well *in vitro*, they were tested *in vivo* on peripheral blood mononuclear cells from children with acute measles. We found 13 up regulated genes and 206 down regulated genes in children at discharge from the hospital and at 1-

month follow-up. All of the up regulated genes peaked at discharge but did not return to baseline by the time of follow-up. The same pattern was found in the down regulated genes. A number of immune factors were up regulated including interleukin-1β, interleukin-8, TNF-α, CXCL2 and CCL4. The down regulated genes were involved in three main biological processes: transcription, signal transduction and the immune response, but also included the chemokine receptors CCR2 and CCR7.

Finally, a vaccine model in mice compared a formalin-inactivated measles vaccine (FIMV) with an alphavirus replicon particle vaccine expressing the H protein (VCR-H) from measles virus. Although both vaccines induced comparable antibody titers to measles virus, VCR-H induced many more interferon-γ producing cells. Gene expression analysis found many genes significantly regulated at day 4 post-vaccination in CD8+ T cells from VCR-H vaccinated mice, while FIMV vaccinated mice did not show any gene regulation until day 28. Many of the genes regulated by both vaccines were involved in transcription and signal transduction.

High-throughput techniques are changing the nature of biological research by providing a new view of the cell. This increased data load requires more computational power and statistical expertise to effectively manage the information and extract knowledge. As scientists learn to harness the power of these new technologies, basic understanding of the cell and the fight against disease will benefit.

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To my Mother,

who taught me more about public health than the world's best school.

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Morbillivirus-Host Cell Interactions

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The Morbillivirus Genus

The morbillivirus genus of negative-stranded, enveloped, RNA viruses causes severe and often fatal disease in a wide variety of mammals, with neurovirulence and immunosuppression salient characteristics. The genus is found within the paramyxoviridae family, which is divided into two subfamilies, the paramyxovirinae and the pneumovirinae. The paramyxovirinae subfamily, in addition to morbilliviruses, also includes respiroviruses and rubulaviruses. Until recently, there were four known morbillivirus members: measles virus (MV), an acute disease of young children, rinderpest virus (RPV), which afflicts cattle and artiodactyls, peste des petits ruminants virus (PPRV), a disease of small ruminants like goats and sheep, and canine distemper virus (CDV), a neurological disorder affecting domestic and wild canines.

In the late 1980's, a large number of harbor seals *(Phoca vitulina*) were succumbing to an infectious disease in the seas northwest of Europe. Initially a team of scientists isolated a herpes-virus and a picorna-like virus from the dead seals, but later concluded these were opportunistic infections[1]. It had been observed that the seals exhibited distemper-like symptoms, leading scientists to search for evidence of canine distemper virus. They found that the seal serum reacted to CDV, CDV-like antigens could be detected in the lungs, and dogs could be experimentally infected with a virus cultured from the seal tissue[1, 2, 3]. The virus was eventually isolated and named phocine distemper virus[4]. Serological studies showed that PDV was more closely related to CDV than to MV or RPV[5].

In 1990, a similar epidemic hit striped dolphins (*Stenella coerruleoalba*) in the western Mediterranean Sea, leading to the identification of dolphin morbillivirus (DMV) [6]. The epidemic continued to spread, and the next year striped dolphins were also found dying in Italy and Greece[7]. An analysis of other marine mammals revealed one more morbillivirus, porpoise morbillivirus (PMV), which was closely related to DMV[8, 9]. Many other marine mammals have been screened for morbilliviruses, but only serological evidence of DMV-like infections in Fin whales (*Balaenoptera physalus*) has been reported[10]. Experimentally, DMV and PMV could infect goats, sheep and cows, causing viremia and leukopenia[11]. Sequence comparisons have shown the DMV is almost equidistant from the other known morbilliviruses, suggesting that it may be most closely related to the ancestral virus[12].

These marine viruses have caused massive epizootics, killing 18,000 harbor seals in 1988 and hundreds of dolphins in the Mediterranean in 1990[6, 13]. Following the outbreaks, the viruses did not seem to be circulating in the wild populations where the epidemics occurred, and therefore may have been transmitted from other aquatic or terrestrial species[14]. Morbilliviruses are spread via respiratory droplets and are some of the most contagious pathogens known, so interspecies transmission may frequently occur[14, 15]. Hooded and ringed seals, which live in the Antarctic, are known to be seropositive for PDV and it is believed that DMV may be spread by bottlenose dolphins, which can be found throughout the world[15]. How and where these carriers transmitted the virus to susceptible populations is unknown. Recently, the finding of a number of stranded seals suggests another outbreak is beginning in 2002[13]. The disease is able to

spread rapidly due to the migratory nature of harbor seals, raising concerns about the possibility of another epidemic.

The terrestrial viruses also cause significant morbidity and mortality, particularly in Africa, the Middle East, and Asia. Rinderpest virus primarily infects cattle, causing major epidemics in developing countries. The RPV strains can readily be grouped into clades depending on the region of origin[16]. Small ruminants succumb to peste des petits ruminants virus, which quickly kills young animals[17]. There is some overlap in the host ranges of RPV and PPRV[17]. Finally, measles virus is one of the great human scourges. Despite being controlled in much of the world, it still takes an enormous toll on children in developing nations.

This chapter will examine the molecular interactions between the morbilliviruses and the host cell as a prelude to the study examining the host response to the virus in Chapter 2. It will begin with receptor binding and follow viral infection through fusion, replication, and finally budding. The next section will cover the known molecular aspects of immunosuppression, a hallmark feature of morbilliviruses and a principal cause of the high mortality associated with infection. Finally, the main issues in microarray analysis will be discussed.

The Cellular Receptor

Identifying the measles virus receptor has been a long and twisted experimental journey. In 1993, Doris and colleagues used human-rodent somatic cell hybrids to test the ability of measles virus to bind to the non-permissive rodent cells[18]. They found that only those cells containing human chromosome 1 were able to bind virus.

Concomitantly, another group in France used a monoclonal antibody that blocked viral infection to isolate 57 and 67 kDa proteins[19]. Amino-terminal sequencing identified the protein as CD46, a complement regulatory protein found on nearly all cells. Alternative splicing and variations in glycosylation lead to 4 different isoforms of the protein[19, 20]. Both groups then expressed CD46 in non-permissive cell lines and were able to show that measles virus could bind to the cells and produce infectious virus. Radioactive labeling proved that new virus was being synthesized. Not all cell lines tested could support viral replication though, suggesting other host components may be involved[19]. These initial studies were conducted with laboratory-adapted strains, and soon it became clear that CD46 was not the only molecule involved.

CD46 is expressed on most nucleated human cells. This expression pattern does not correlate well with the known tropism of measles virus, which prefers cells of the immune system for infection, but can also infect epithelial and neuronal cells. In addition, it was found that wild-type isolates did not grow on cell lines, such as vero cells, known to express CD46. Laboratory and vaccine strains were able to grow on these cell lines, but wild-type isolates could only be maintained on primary cells or B95a's, a marmoset B cell line. CD46 is down regulated following infection with vaccine or laboratory strains, but that this did not occur with wild-type strains[21]. Data such as this convinced several groups that there was more to the MV receptor story.

Again, two separate research groups were able to identify another receptor at nearly the same time. Knowing that B cell lines, including B95a cells, were able to support wild-type virus replication, a Japanese team lead by Y. Yanagi reasoned that these cells contained the receptor coding mRNA. They transformed the human kidney

cell line 293T's, which can be infected with Edmonston but not wild-type strains of MV, with cDNA from B95a cells. The cells were infected with a VSV construct that replaced the VSV G gene with green-fluorescent protein and contained the MV haemagglutinin from the KA wild-type strain and the fusion protein from the Edmonston strain. Transformed cells that could support MV binding and entry were identified by fluorescence. The group enriched the fluorescent cells until they obtained a single clone and then sequenced the B95a cDNA. The DNA insert was 91% identical to the human signaling lymphocyte activation molecule (SLAM), which is also known as CD150[22].

To confirm the findings, the group first transfected Chinese hamster ovary cells with human SLAM. Then they tested a number of VSV constructs and MV isolates for the ability to infect the transfected cells. Wild-type MV could infect the SLAM expressing cells, but not CD46 expressing cells, while the Edmonston strain could infect either cell line. Infectious virus was recovered from these cells at 36 hours[22]. In addition, the wild-type strains induced cytopathic effect (CPE) on the SLAM expressing cells, but not untransfected or CD46-transfected cell lines. A monoclonal antibody against SLAM, IPO-3, blocked wild-type infection and subsequent CPE. Raji, Ramos, BJAB-B95-8, MT-2, and C91/PL are all cell lines known to be susceptible to clinical isolates of MV and immunofluorescence showed they all expressed SLAM on their surface[22]. The non-permissive cell lines, BJAB, Daudi, Jurkat, and MOLT-4 did not[22]. Finally, the group tested 6 clinical isolates and found that they all could infect SLAM-expressing CHO cells, induce CPE, and the infection could be blocked with IPO-3[22].

Expanding on this work, the group also analyzed receptor usage by other members of the *Morbillivirus* genus[23]. They cloned the canine and bovine homologs of

SLAM and transfected CHO cells with them. Wild-type isolates of canine distemper virus and rinderpest virus could infect the transfected cells but not untransfected controls. In addition, the viruses could infect cells expressing SLAM from other species. Again, the wild-type isolates caused CPE in transfected cells and infection was blocked by anti-SLAM antibody[23].

Erlenhoefer et al. confirmed SLAM as a MV receptor using an alternative approach. They raised antibodies against the surface proteins of B95a cells and selected one that blocked infection with a wild-type strain. In a separate experiment, they also biotinylated B95a cells and incubated them with MV overnight. Then they precipitated the virus-receptor complexes with an antibody directed against MV H. Running the precipitates on an SDS-gel, they concluded that the proteins pulled down by the two antibodies were the same size. To determine the identity of the protein, they used the antibody to screen CHO cells transduced with a human splenocyte library. With this technique they independently identified SLAM as a measles virus receptor[24]. They also found that SLAM was down-regulated from the surface of PBMC's, B95a, and BJAB cells following MV infection, leading them to speculate that receptor-binding may play a role in MV-induced immunesuppression[24].

SLAM, a 70-kDA membrane glycoprotein, is a member of the CD2 subset of the immunoglobulin superfamily and is found on immature thymocytes, T cells, memory T cells, some B cells and mature DCs[24, 25, 26]. The cellular expression of SLAM correlates better with the known tropism of MV than the ubiquitous CD46. SLAM is its own ligand and is rapidly induced on T cells, B cells, monocytes, and DC's following activation[25, 26,

[27]. SLAM promotes T cell proliferation and IFN-γ production through a CD28-independent pathway and may help to promote the cell-mediated immune response[28].

As its name implies, SLAM is involved in lymphocyte signaling, particularly in bi-directional activation between B and T cells, leading several groups to speculate that MV-induced immune suppression may in part be mediated by SLAM engagement[22, 24, 29]. In fact, X-linked lymphoproliferative disease (XLP) and some misdiagnosed cases of hemophagocytic lymphohistiocytosis result from defects in SLAM signaling[25, 30]. Upon CD3 signaling, SLAM colocalizes with the T cell receptor where it is phosphorylated by the Src-like kinases Lck or Fyn[31]. It then binds with SAP/SH2D1A, the defective gene in XLP[29]. Activation of the SLAM signaling pathway redirects Th2 responses to a Th1 profile and induces interferon-γ production.

Further studies have supported the finding that SLAM is the receptor for wild-type measles virus. The Yanagi group followed up their receptor identification paper with an analysis of 9 clinical MV isolates. Seven of the nine isolates could infect vero cells transfected with SLAM cDNA, but none of the isolates could infect untransfected vero cells[32]. In a similar study, Erlenhoefer et al. used Chinese hamster ovary cells expressing CD46 or SLAM to test 28 viral strains for binding and replication. They found that all of the strains tested could infect the SLAM-expressing cells, but only the laboratory, vaccine, and a few of the wild-type strains could infect the CD46-expressing cells[33]. They went on to show that a single amino acid change at position 481, from an Asn to a Tyr, of the haemagglutinin (H) protein was sufficient to confer CD46 binding to a strain[33]. Binding to SLAM was not affected by the mutation. The MV H protein is a type II transmembrane glycoprotein that is important for viral tropism[34]. It is responsible

for the receptor binding and haemagglutinating properties of the virus, and unlike many other paramyxoviruses, the H protein of measles does not possess neuraminidase activity. The protein has not been crystallized, but sequence alignments with MV H, influenza N, and parainfluenza HN predicted a structure with a globular head sitting atop a stem and transmembrane domains[35]. The globular head is made of six antiparallel β-pleated sheets, which fold to form a superbarrel structure[34]. On the cell and virion surface disulfide-linked homodimers of H form tetramers[36].

The MV receptor story may not be finished. Although the evidence strongly supports the use of SLAM by wild-type viruses during natural infection, it still does not explain how the virus enters SLAM-negative cells such as epithelial, endothelial, and neuronal cells[37]. A recent report showed that the virus could enter SLAM-negative cells and replicate, but 2 to 3 logs less efficiently than infection of SLAM-positive cells[37]. Continued efforts at understanding receptor usage and viral entry will help to clarify the natural course of infection.

Viral Attachment and Fusion

Measles virus is a negative-stranded virus that acquires its viral envelope from the host plasma membrane. The matrix (M) protein lines the inner layer of the envelope while the haemagglutinin (H) and fusion (F) proteins are exposed on the outside of the virus particle. Their appearance has been described as that of spikes protruding from the surface. To gain entry into a host cell, these proteins must interact with the host plasma membrane (PM), fuse the viral envelope to the PM, and deliver the viral genome and proteins into the cytoplasm. The F protein mediates this pH-independent process.

The type I transmembrane F protein is 550 amino acids long and has a molecular weight of 60 kDa[36, 38]. It was shown in Sendai virus that this protein contains the hemolysis, cell fusion, and infection activities of the virus[39]. However, the protein is synthesized as an inactive precursor protein[40]. The F protein is proteolytically activated in the Golgi or trans-Golgi network[41]. The enzyme responsible for cleaving F into its disulfide-linked polypeptides, F_1 and F_2, is believed to be furin, a subtilisin-like protease[42]. Following cleavage and activation in the trans-Golgi, F_1-F_2 is transported to the cell surface where it is found as a trimer and may or may not associate with the H protein[36].

Paramyxovirus F amino acid sequence comparisons reveal very little homology, except at the F_1 amino terminus along with nine conserved cysteines[38]. Cysteines at amino acids 506, 518, and 524 are palmitoylated while those at 506 and 519 were found to be particularly crucial for the fusion process[43]. All of the fusion proteins examined had an overall hydrophobic nature[38]. Also, the F mRNA has a long, G-C rich non-coding region at the 5' terminus that may play a role in the regulation of protein synthesis[38]. Computer modeling also suggested that long stretches of α-helices were responsible for membrane interactions during fusion[38]. Some of these features are also found in HIV gp120 and influenza HA[44].

The F protein contains two 4-3 heptad repeats, characterized by hydrophobic residues in recurring positions, next to the fusion peptide and the transmembrane domain that may be important for inhibiting fusion[44]. The protein is found as a trimer on the surface of cells and is believed to form a six-helix bundle during the fusion process[44]. Lateral mobility is required for both F and H in order to undergo fusion[45]. The fusion

peptide, leucine zipper, transmembrane region, and cytoplasmic tail of F are not important for its interaction with H[46]. The region of importance was narrowed down to the cysteine rich region of the F protein, which has long been suspected to play a role in the fusion process[46].

The H protein is the main determinant of viral tropism based on receptor binding, but its role in viral fusion is still contentious[47]. There are three main ideas: 1) The H protein is not strictly required. Through receptor binding it helps to bring the viral membrane within close proximity of the host PM, thereby increasing fusion efficiency. 2) The H protein interacts with the F protein and, upon ligand binding, induces a conformational change in F, which exposes its hydrophobic domain. 3) Binding of H to the receptor sends a signal to the host cell or the virion to initiate the fusion process[45]. There is little experimental evidence in support of the third idea, but a wealth of data support both the first and second ideas.

Experiments with micelles containing the F protein showed that fusion is possible if an agent is added to bring individual micelles together[48]. Also, certain paramyxovirus F proteins, when expressed alone, can induce fusion. These include SV5, peste des petits ruminants virus, and MV F expressed in an adenovirus vector[49, 50, 51]. In most of these examples, coexpression of the H protein often enhances fusion and may be its main determinant[52]. However, other paramyxoviruses have required both H and F to be expressed to induce fusion, such as Rinderpest virus, NDV, and SV41[50, 53, 54]. Using a vaccinia virus construct, Wild et al. showed that for measles virus, both H and F are required for fusion[55]. Human parainfluenza virus 2 (hPIV2) also required both proteins for fusion, although they did not have to be expressed on the same cell surface[54, 56]. The

most likely explanation for the disparate results lies in the different expression systems used[57].

A salient feature of fusion is that when H is required, it must be from the same virus as F. Using H from one virus and F from a closely related virus does not usually result in fusion. Evidence like this suggests that H does play an active role in the fusion process and does not simply bring the membranes close together. Researchers have exploited this phenomenon and used it to produce H protein chimeras. With this strategy, hPIV2 researchers have narrowed the region of H necessary for fusion down to the transmembrane and the stalk regions[54]. Interestingly, the globular head does not play a role[54].

Further evidence from mutational analyses also supports the role of H in the fusion process. The H protein is 617 amino acids long and contains one hydrophobic transmembrane domain[58]. It naturally occurs as a tetramer on the surface of the host cell[59]. Studies with human parainfluenza virus 3 (hPIV3) and Newcastle disease virus (NDV) showed that this interaction is important for fusogenicity. The specificity of the interaction resides in the transmembrane region and first 82 amino acids of the ectodomain, but not in the cytoplasmic tail or globular head[60]. This was also observed in Sendai virus[61]. In addition, conserved leucines in heptad-repeat motifs in the transmembrane domain are important for tetramer formation, and mutation of these conserved residues resulted in decreased fusogenic activity[62].

In contrast, studies in measles virus have shown that the cytoplasmic domain of H may play a role in fusion. Moll et al. found that the cytoplasmic tail of H could only be truncated to 14 residues before it began to affect the ability of H to augment the fusion

ability of F[63]. The cytoplasmic tail of F was not important though, as it could be truncated to 3 amino acids without adversely affecting fusion. It was also noted that the tyrosine residue at position 481 is particularly important for cell fusion[64]. By adding an eight amino acid tag to the H protein of MV, Plemper et al. were able to diminish the interaction between H and F on the host cell surface[65]. Surprisingly, this weaker interaction resulted in increased and earlier syncytium formation[65].

Also supporting an active role for H in membrane fusion is the identification of monoclonal antibodies to NDV and Sendai virus that block receptor binding but do not inhibit fusion[66, 67]. The studies in NDV showed that some antibodies could even differentiate virion-cell from cell-cell fusion[66]. Additionally, the receptor has been found to play a role in fusion. Studies with CD46 showed that by varying the length of the receptor, researchers could increase viral binding while concomitantly decreasing fusion[68].

Seemingly at odds with other evidence, and in support of the hypothesis that H triggers a conformational change in F, experiments with NDV identified a point mutation in the globular head of H that disrupted receptor recognition, neuraminidase activity, and fusion activity[45, 69]. The mutation also disrupted H and F interactions at the cell surface, as shown by coimmunoprecipitation experiments. The authors argued that if receptor recognition triggers interaction with F and fusion, then a mutation that abolishes receptor recognition should also abolish fusion, which these experiments have shown[69]. If H is only needed to bring the membranes in close proximity, then disrupting the interaction between H and F should not affect fusion.

The physical and biological properties involved in fusion are not yet fully understood. Most of the research conducted has used syncytia induction as its final read out, which is useful for understanding cell-cell fusion, but may not be entirely relevant for virus-cell fusion. In fact, studies have shown that the two processes are not entirely identical[66]. Although the role of H in fusion has not been conclusively proven experimentally, the majority of data suggests it does more than simply bring the two membranes into close proximity. What that role is exactly remains controversial. Because the fusion of two lipid bilayers is such a fundamental biological process, research in this area will continue until scientists have a firm grasp of the underlying principles.

Viral Replication

The essence of viral replication is to produce infectious progeny. For RNA negative-strand viruses, it is a process that requires two distinct phases, both of which occur in the cytoplasm[70]. In the first phase, the genome is transcribed into functional mRNAs. Once accomplished, the virus now faces the task of replicating its genome through a positive strand intermediate. To prevent annealing between the positive strand and the nascent genome, the RNA is always associated with the N protein[71]. The replication machinery of RNA viruses also has very little proofreading activity, causing RNA viruses to mutate up to 10,000 times faster than organisms that use DNA[71]. The high mutation rate ultimately restricts the size of the viral genome, with most RNA viruses having genomes between 5 and 15 kilobases[71].

For morbilliviruses, the replication complex is believed to be composed of an association between the phosphoprotein (P) and large (L) proteins, with all of the catalytic activity contained in the L protein[72]. The MV L protein is composed of 2183 amino acids that are highly conserved among negative strand viruses[36]. The P protein is only 507 aa and is activated by phosphorylation[36]. For measles virus, the major cellular kinase responsible for phosphorylation is casein kinase II[73]. The L-P binding site has been mapped to the N-terminal 408 amino acids of the L protein, which contains the first of six conserved domains[74]. The amino terminal of the P protein is also important for binding to L[36].

As noted, the L protein is highly conserved among the negative strand viruses and contains glycine-asparagine-asparagine (GDD) residues flanked by strong hydrophobic regions, which are believed to be remnants of an ancestral RNA polymerase[75]. Sequence comparisons between morbillivirus L proteins have shown that MV and Rinderpest virus are closely related, as are CDV and PDV[76]. The comparisons have also revealed six highly conserved domains separated by less conserved regions[77]. Three of the domains in the N-terminal half of the protein are conserved amongst the paramyxo-, filo-, and rhabdoviridae[78]. The conserved domains are believed to contain the enzymatic functions of the protein. The carboxy terminus displays higher variability and may contain virus-specific functions[79].

The L proteins are unusual in that they have high leucine/isoleucine content and are basic[80]. Domain I is important for binding to the P protein, which may in turn regulate binding to the RNA-N template[81]. Studies in Sendai virus have shown that mutations at amino acid 368 and a three amino acid insertion at 379 almost completely

disrupt complex formation and RNA synthesis[82]. Domain II contains a charged, RNA binding motif[81]. The functions of the remaining domains have not been fully characterized. The domains have been extensively mutated, with various effects on RNA synthesis, but the precise function of each domain has remained unclear[81].

The nucleocapsid protein must be bound to the viral RNA in order for transcription and replication to proceed. The N protein is 525 amino acids long and is the most abundant viral protein due to its 3' position on the negative-sense RNA[36]. It is believed to interact with genomic RNA, the P protein, and itself in order to form the ribonucleoprotein complex[83]. Following synthesis on free ribosomes, the N protein is folded and rapidly incorporated into the viral nucleocapsid[84]. Deletion analysis has shown that amino acids 4-188 and 304-373 are required for the formation of the N-P complex[85]. In order to achieve efficient replication of the morbillivirus genome, the total length must be a multiple of six. The rule-of-six is presumably due to the functional binding unit of the nucleoprotein, which is six nucleotides[70]. The association of each N protein with the RNA chain probably begins at the 5' end and continues to the 3' end. If the genome is not a multiple of six, a few nucleotides will overhang at the 3', preventing efficient initiation by the polymerase[70].

The viral RNA polymerase transcribes the first strand leader RNA followed by the viral RNAs in the following order: 3'-N-P+C-M-F-H-L-5'[86]. The L protein is responsible for capping, methylating, polyadenylating, and editing all of the mRNAs[81, 87]. As transcription proceeds, the RNA polymerase moves from one coding region to the next, but occasionally falls off and does not reinitiate at downstream gene junctions, generating an mRNA gradient from 3' to 5'[88]. The relative mRNA amounts compared to

N have been quantified to be P (81%), M (67%), F (49%) and H (39%)[772]. The RNA polymerase activity is dependent on NTP precursors and divalent cation[89].

Following transcription, the genome is replicated via the antigenome, its complementary copy. The switch from transcription to replication is due to an accumulation of free N protein[70]. To synthesize the antigenome, it is thought the same polymerase copies the same template, but ignores the gene junction signals[70]. Synthesis of the genome from the antigenome is thought to occur by a similar process, but there are no reinitiation sites on the antigenome, so replication proceeds smoothly. The leader sequence also plays an important role in the regulation of transcription and replication. Most of the monocistronic N mRNAs lack the 55 nucleotide-long leader sequence, while all of the plus-strand genomes contain it[90]. This led to the hypothesis that the leader RNA determines whether RNAs are destined for encapsidation or translation. It has also been proposed that the rapid accumulation of the leader sequence may play a role in down-regulating host cell transcription[91].

Host cell proteins appear to play a role in measles virus replication, although they have not been clearly defined. Actin has been detected in the virion and studies in human parainfluenza virus 3 (hPIV3) have shown that NP and P interact with the cytoskeletal framework[92]. Treatment of cells with cytochalasin D, which disrupts the cytoskeletal network, diminishes RNA synthesis by 70%[92]. Actin may play a role in the budding process by dragging the assembled ribonucleoprotein to the surface during filament elongation. Cell-free transcription systems with measles virus have shown that β-tubulin is required for efficient transcription and replication *in vitro*, although coimmunoprecipitation experiments showed that α-tubulin associated with the L

protein[93]. These results were also observed in VSV and Sendai virus, but have not been shown *in vivo*[93]. Further studies have also identified host cell proteins that interact with the genomic 3' noncoding region and the plus strand leader sequence. Using RNA footprint analysis, Leopardi et al. have shown that a 20 kDa protein binds to the 3' noncoding region and 22 and 30 kDa proteins interact with the leader sequence, but the proteins have not been identified[94].

The P mRNA is polycistronic and encodes three other non-structural proteins- C, V, and R[36, 95]. The V protein is produced by RNA editing, where the viral RNA polymerase inserts a single, non-templated G residue following a string of 3 genomically encoded G residues at position 751[36, 96]. Therefore, the V protein has the same 231 amino-terminal residues as the P protein, but contains a unique 68 amino acid carboxy terminus. The amino terminal region contains the phosphorylation sites and one of the two N-interaction sites[97]. The unique carboxy terminus contains seven highly conserved cysteine residues, which form a zinc binding domain[98]. Yeast two hybrid experiments failed to find an interaction between V and N, suggesting rather that V interacted with 17, 38, and 60 kDa host cell proteins, but other studies have found an interaction between V and N necessary to balance viral transcription[97, 99]. In rinderpest virus, V interacts with both N and L[100].

The C gene initiation site is encoded 19 nucleotides downstream of the P gene start site in the +1 reading frame[36]. Although measles virus only encodes one C protein, other paramyxoviruses encode up to four, and use alternate start codons[101]. Like the V protein, it is unclear if C interacts with other proteins. The C protein has been shown to form complexes with host cell proteins, and in RPV, to interact with the L protein[99, 100].

The different assays and cell systems used are the most likely explanations to account for these observations. The precise functions of the V and C proteins have remained elusive.

The C and V proteins are not strictly required for measles virus replication *in vitro*, but mutations or deletions in either gene can affect viral RNA synthesis *in vivo*[97, 102, 103]. In both MV and RPV, defects in the V protein lead to increased synthesis of viral RNA and proteins while also increasing syncytium formation[97, 104]. Without the C protein, viral RNA synthesis is reduced in MV-infected PBMC's or in RPV-infected cell lines[104, 105]. *In vivo*, V- or C-deficient viruses replicate slower and show less pathogenicity, suggesting the proteins may play a role in avoiding host defense systems[97, 106]. Interleukin-1β, IL-12, and TGF-β levels were comparable between mutant and wild-type viruses, but TNF-α and RANTES levels were lower in mice infected with mutant virus[103].

Once transcription has produced mRNAs, they are translated by the host ribosomes. The final protein products must then be sorted to the proper intracellular location, processed, and transported to the plasma membrane. Likewise, the nascent viral genomes must also find their way to the cell surface in order for assembly to proceed.

Protein Sorting and Interaction with Lipid Rafts

Once measles virus has completed its genome replication, it faces the task of assembling its ribonucleoprotein complex, consisting of the genomic RNA along with the N, P and L proteins. The RNP and remaining structural proteins, M, H and F, must then be transported to the plasma membrane where they associate to produce a mature viral bud. The efficiency of nucleocapsid formation and the final structure may be regulated

by the P protein[107]. Several lines of evidence suggest that the main mediator of viral transport and assembly at the plasma membrane is the M protein.

The morbillivirus M gene is 335 amino acids long and has a molecular weight of 38 kDa[108, 109]. The carboxy-terminal third of the protein is most highly conserved and contains a hydrophobic sequence, which may interact with the lipid bilayer[109, 110]. The M protein is thought to have two or three functional domains; one to interact with the inner leaflet of the plasma membrane, another to interact with the nucleocapsid, and possibly a third that binds to actin. The M protein interacts with the nucleocapsid across the plasma membrane at the cell surface[111]. This interaction was blocked by cytochalasin B, suggesting a role for actin in the process[111]. The M protein also interacts with the viral RNP, which may inhibit RNA transcription[112]. The role of M in protein sorting and assembly is complicated by the pathogenesis of the virus.

Measles virus is spread via respiratory droplets and initially infects and replicates in respiratory tract epithelial cells. In addition, in order to spread, the virus must also find its way back into the lung expirate so that it can be transmitted from individual to individual. Because epithelial cells are polarized, there may be viral mechanisms for entering the apical surface of lung epithelium upon infection. Likewise, the virus may induce the cell to preferentially sort it to the apical or basolateral surface following infection.

This question was first addressed in the polarized vero cell line C1008, a monkey kidney line, and Caco-2 cells, a human intestinal epithelial cell line. In both cell types, virus added to the apical side of the cell was able to replicate much more efficiently than virus added to the basolateral side[113]. Virus release was also polarized, with titers from

the apical medium being 1000-fold higher than those from the basolateral medium[113]. This finding was later extended to the Madin-Darby canine kidney (MDCK) cell line, the best-studied cell line for polarized sorting of proteins[114]. Repeated passages of the virus induce more efficient release from the basolateral side[115]. When primary cultures of well-differentiated human airway epithelial cells were used, it was found that the virus preferentially transduces the basolateral side and is released from the apical side[116]. The reason for this discrepancy is unclear, but it may be related to the surface expression of the viral receptors. The CD46 receptor is preferentially expressed on the apical side of C1008, Caco-2, and primary epithelial cells, while the expression of SLAM in polarized cells has not been studied yet[113, 116].

For many viruses, polarized release is thought to relate to the cellular localization of the envelope proteins, although measles virus appears to have developed a strategy that is different than that used by other viruses[117]. Following translation, H and F are folded by chaperones in the endoplasmic reticulum, where they first associate, forming hetero- and homo-oligomers[118, 119]. In Sendai virus, the M protein binds to H and F as it transits through the Golgi network, although this has not been shown for measles virus[120]. As they progress through the sorting pathway, F is proteolytically cleaved into F_1 and F_2 in the trans-Golgi prior to sorting. Measles virus H and F are then found on the apical and basolateral membranes of MDCK and CaCo2 cells[114, 121]. Although the virus buds apically, basolateral expression of the glycoproteins may help the virus to spread by cell-cell fusion. In support of this idea it was found that both H and F contain a basolateral targeting signal, which can be overridden by mutating a single tyrosine in the cytoplasmic tails, amino acid 12 in H and 549 in F[122]. In addition, the cytoplasmic tails of H and F

could redirect the influenza H7 protein, which normally sorts apically, basolaterally. These signals also serve to initiate endocytosis, although viral infection appears to block this function[122].

Since H and F contain intrinsic basolateral sorting signals, they must interact with other viral proteins in order to bud apically. Studies of virus isolated from subacute sclerosing panencephalitis patients (SSPE) revealed the role of the M protein in assembly and budding. SSPE viruses are usually defective in virion production and spread by cell-cell fusion[123]. Most viruses isolated from SSPE patients had a defective M protein and altered cytoplasmic tails in their fusion proteins[124]. These defects abrogated binding between M and the viral nucleocapsid[123, 125]. Several of the SSPE mutants have shown a biased hypermutation where the most common mutation is often a U to a C[126]. It was found that the M protein interacts with the cytoplasmic domain of F and when defective M proteins were incorporated into virions, increased fusion was observed, which is associated with basolateral sorting[127, 128]. Additionally, studies in polarized epithelial CaCo2 cells showed that in the absence of M the envelope glycoproteins preferentially sort to the basolateral surface. When the M protein was added, the glycoproteins were found on the apical side[121]. The M protein preferentially associates with the apical side of MDCK and CaCo2 cells, but in transfected cells M is not found at the plasma membrane[121]. This suggested other factors present in a full infection were needed for M to be transported to the plasma membrane[129].

The additional factors may include components of the cytoskeleton, particularly actin. Early transmission electron microscope studies showed viral particles associated with actin filaments, with 76% of the filament's barbed ends protruding into budding

virus[130]. Observations like these led the authors to speculate that the vectorial growth of actin filaments played a key role in viral budding. Studies in Sendai virus have supported the idea that actin is involved[131, 132]. Wild-type Sendai virus buds apically from MDCK cells, but a mutant, F1-R, buds in a bipolar fashion[131, 132]. Both glycoproteins are found apically and basolaterally. Treatment of the cells with depolymerizing drugs also resulted in bipolar release of mature virions, suggesting the mutation in the F1-R strain results in a defective interaction between the M protein and the cytoskeleton[135]. Blocking actin filaments in MV-infected cells leads to a drastic decrease in the production of infectious virus[134].

Membrane proteins are sorted in the trans-Golgi network for targeting to the plasma membrane or intracellular compartments, depending on the signals they contain. N-glycans and GPI anchors can be used as apical sorting signals while basolateral signals originate from the cytoplasmic domains of proteins[135]. While it is known that basolateral transport uses the same machinery as synaptic vesicle transport and ER-Golgi transport, the mechanisms used for apical transport are not well known. The ribonucleoprotein complex, which consists of the genomic RNA, nucleoprotein, viral polymerase, and the phosphoprotein, assembles in the cytoplasm. The RNP must then enclose itself in a host-derived membrane that is enriched in the M, F, and H proteins to initiate viral budding. It is thought that M interacts with actin, which is also found in the virion. By interacting with actin, the growth of actin filaments can transport the RNP from the cytoplasm to the PM, but whether or not the transport is mediated by M has not been shown[72]. The matrix protein, which lines the inside of the membrane, binds to the cytoplasmic tail of F and may concentrate the F and H proteins at the assembly site to facilitate budding.

The location of viral assembly and budding has been postulated to be lipid rafts so that viral proteins can be concentrated while host proteins are selectively excluded. To support these ideas, recent research has shown that the M, N, H, and F proteins are selectively enriched in lipid raft regions of the PM[136]. The M protein may act as the organizing center for viral budding since it appears to concentrate the H and F proteins along with the ribonucleoprotein complex at virus assembly sites. Early structural studies showed that viral assembly probably occurs at the PM[137, 138]. When lipid raft regions are isolated, infectious virus can be recovered. Gerlier et al. found that 15-40% of H and F colocalize with the raft fragments, while 35% of M and 25% of N did so[136]. The nonstructural V protein, as a control, was predominantly recovered from the soluble fractions. Further studies showed that M and N could attach to rafts independent of interactions with H or F[136]. Time course studies revealed that maximal attachment of raft proteins occurred between 4 to 6 hours following synthesis[139].

Although a lot of research has been done on intracellular sorting, transport, assembly, and budding, many questions remain. Conflicting results have been published regarding polarized epithelial cell sorting, and the *in vivo* situation remains unclear. The actual cellular machinery involved in apical sorting has yet to be characterized, and studies of viral protein sorting pathways may help illuminate this aspect of cell biology. Finally, the biology behind budding is only now beginning to be understood. The recent discovery of lipid rafts has invigorated the field, and should help clarify this aspect of the viral life cycle.

Alteration of Cellular Immunity

Measles virus is renowned for its ability to suppress the immune response of the host organism. The high mortality associated with measles is attributed to the immunosuppressive effects of the virus and the host's subsequent susceptibility to secondary infections. Vaccination also results in a suppression of the DTH response, although it is much more mild[140]. The cellular and molecular mechanisms behind the immune suppression are coming to light, but it is a complicated problem.

The finding that monocytes are a main target of the virus *in vivo* and *in vitro* opened the door to more thorough studies of the immunesuppression[141, 142]. In addition, neonatal monocytes were noted to be particularly susceptible[143]. It was subsequently discovered that measles virus infection specifically down-regulated IL-12 production from monocytes, which was replicated by cross-linking the CD46 receptor[144]. It has been suggested that monocytes and macrophages serve as a barrier to protect DC's, which present MV antigens most efficiently, from becoming infected and also serve as vectors to transport the virus from the periphery to regional lymph nodes[145, 146, 147]. The maturation state of the cell also influences susceptibility, as monocyte precursors are readily infected and produce higher amounts of infectious virus[148]. It was also found that maturation leads to cellular changes that block viral replication posttranscriptionally and posttranslationally[148]. These results do conflict with the known expression of SLAM, which is found primarily on activated and mature cells, although precursor cells have not been specifically looked at. It is also generally accepted that activated cells are more permissive to the virus[149].

For over two decades it has been known that MV infection can disrupt the cellular production of the type I interferons, interferon-α and β, which are the main anti-viral cellular defense. In 1977 it was shown that attenuated strains of the virus induced significantly more interferon than wild-type strains[150]. The low production of interferon led to high viral titers[151]. Later studies showed that wild-type infection partially blocked STAT1α and STAT1β production, which are in the interferon α/β signaling cascade[152]. If cells are preinfected with wild-type virus, they can block interferon production by subsequent infection with attenuated virus, but not dsRNA-induced interferons[152]. In mice transgenically modified to express CD46, the interferon α/β receptor needs to be knocked out in order to get efficient spread of the virus, demonstrating the potent effects of this response[146]. Sendai virus also blocks interferon production, particularly through its AUG[114]-initiated C protein[153]. The mechanism is believed to be through inhibition of an α/β responsive promoter although a direct link has not been established[154]. SV5 also inhibits the promoter in human cells[154].

A second facet of the immune suppression is that viral infection blocks the mitogen-stimulated proliferation of lymphocytes in culture. The virus appears to block the cell cycle progression at the G_1 phase in both lymphocytes and thymic epithelial cells[155, 156]. The effect on thymic epithelial cells may be particularly important in children, who still have a developing immune system. All members of the morbillivirus family block proliferation and it has been shown with measles virus that expression of the H and F proteins alone is sufficient[157]. Fusion with the host cell membrane is not required[158]. It has also been suggested that there is a separate suppressive pathway that only requires the interaction between H and CD46, but this may not occur *in vivo*[159].

Another mechanism has also been identified in T cells where MV infection has been shown to block the IL-2 pathway by blocking the activation of Akt kinase[160].

In addition to blocking proliferation, morbilliviruses may also induce apoptosis in infected cells. When SCID mice with grafted human thymuses were infected with virulent measles virus, there was a 100-fold decrease in the number of viable thymocytes[161]. This study also showed that thymic epithelial cells were infected[161]. Peste des petits ruminants virus induces apoptosis in goat PBMC's[162]. In addition to the thymus, the bone marrow may also be infected during measles, supporting the idea that immature cells are susceptible[163].

The mechanisms of immune suppression are complex and multifaceted. New technologies have the potential to provide important clues for understanding the complicated interactions between the virus and the host immune system. By deepening our knowledge of this complex effect, scientists will gain insight into many areas of biology, and hopefully this insight will help to find new strategies for combating these devastating viruses.

Microarray Analysis

Microarrays are one such technology that gives scientists a new view of old problems. Due to the large amount of data generated, harnessing the power of microarrays requires a lot of computer power and the proper use of statistics. This next section will briefly review the Affymetrix GeneChip® system and the statistical methods that provide the best results to date.

The Affymetrix GeneChip® system uses approximately eleven 25-oligomer probes scattered across a gene to measure the expression level of that gene. For each probe there is a perfectmatch (PM) and mismatch (MM), which differs from the PM at the 13th nucleotide to serve as a background binding control. The probe values are then assembled to determine a gene expression value (referred to as signal intensity in the normal Affymetrix protocol). The signal intensity is usually normalized and examined for expression differences, but it has been shown that normalizing probe level data can be advantageous[164].

Normalization is an important consideration when analyzing gene array data in order to minimize the variation due to non-biological variables (i.e. the scanner) and to maximize the detection of biological variability. The standard Affymetrix normalization does not work well with nonlinear relationships between chips, which often occur in gene array experiments[165]. Several normalization options exist, see Bolstad et al. for a more complete review, but quantile normalization is preferred because it is robust, computationally fast, and does not rely on choosing samples for a baseline[165, 166]. Quantile normalization does assume that a small percentage of the genes are significantly changing, which has been observed in many Affymetrix experiments and specifically in DC maturation experiments[165, 167].

Following quantile normalization, probe intensities were assembled into gene expression values with Robust Multi-Array Analysis (RMA), also developed by Irizarry et al.[168]. Again, a number of options exist to assemble probe level data, most of which incorporate both the PM and MM probe values, either as PM-MM or PM/MM. Experiments have shown that as the target gene concentration increases the MM

expression value also increases, demonstrating specific binding[169]. One study found over 80% of the mismatch signal, especially at higher concentrations, is from interactions with target transcripts[170]. Li and Wong reached a similar conclusion[164]. Therefore, RMA does not include MM values in the calculations, resulting in a similar accuracy but better precision than some commonly used algorithms as well as providing more consistent estimates of fold change[165, 169]. The differential expression calls are also more specific and sensitive, especially at lower fold change values. For a more thorough discussion, please see Irizarry et al. and Li et al[164, 165, 169].

Chapter 2

Gene Expression in Measles Virus-Infected Dendritic Cells Compared to Other Pathogens

Michael J. Zilliox, Giovanni Parmigiani and Diane E. Griffin

Abstract

Gene expression patterns supply insight into complex biological networks that provide the organization in which viruses and host cells interact. Manipulation of this network by viral proteins causes pathogenesis following infection. In this report we examine the gene expression changes that occur following dendritic cell infection with measles virus over the course of 24 hours. There were 1553 significantly regulated genes with nearly 60% of them down regulated. The dendritic cells up regulated many expected genes such as interferons, interleukins, chemokines, and other antiviral agents, while down regulating genes required for protein synthesis and oxidative phosphorylation. The histone, metallothionein, and insulin-like growth factor binding protein gene families were identified as significantly up-regulated genes that have not been well characterized in infection models. The measles virus-infected dendritic cell results were then compared to eight other pathogen-host cell systems. These results provide a modular view of dendritic cell biology in response to infection.

Introduction

Measles virus (MV) is remarkable for its ability to induce a generalized and prolonged immune suppression concordant with a productive, specific immune response following infection. Measles virus-specific immunity is life-long while the transient immune suppression is responsible for most of the mortality associated with measles virus, which still claims 770,000 children each year[1]. This is particularly troublesome considering the existence of a safe, effective vaccine. However, immunological barriers, including maternal antibody and a developing immune system, prevent effective vaccination of infants in endemic areas before 9 months of age[2].

Viral transmission is via respiratory droplets. The initial replication occurs in lung epithelial cells before the virus spreads to local lymphoid tissue to replicate in macrophages and monocytes, eventually reaching the blood[2, 3]. It has been suggested that dendritic cells (DCs) play a role in transporting the virus from the lungs to the lymph nodes, but it has not been demonstrated that DCs are infected *in vivo*[4, 5]. In support of this idea, DCs generated from $CD34^+$ cells, monocytes, or Langerhans cells can be infected with laboratory, wild type, or chimeric strains of MV *in vitro*[6, 7, 8]. However, it has also been shown that as promyelocytic and promonocytic cells mature there is a decreased production of infectious virus, which is also seen in DCs[9, 10].

Dendritic cells are the most potent initiators of the immune response, but some evidence suggests these functions may be compromised during infection. In culture, CD40-induced DC maturation was altered by MV infection, which inhibited CD40-CD40L signaling between DCs and CD8+ T cells[7]. Infected DCs also produce low amounts of interleukin-12 (IL-12), inhibit mitogen-dependent proliferation of peripheral

blood mononuclear cells (PBMCs), and fail to stimulate T cells in mixed lymphocyte reactions[6, 10, 11]. Reports differ on whether the virus must replicate in order to impair DC function[6, 12]. Measles virus may also induce Fas-mediated apoptosis in DCs to facilitate viral release, while up-regulating tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) to induce apoptosis in lymphocytes[10, 12, 13, 14].

Gene array analysis is a powerful technique that provides a global view of gene expression patterns, but thus far has mainly illuminated the role of individual genes. Specifically regulated pathways will most easily be revealed by gene expression comparisons between similar systems. Comparisons between labs are currently hindered by the wide variety of array platforms, incomplete datasets, and the analysis techniques used. Despite these limitations, we show that meaningful comparisons can be made which elucidate biological insights.

Materials and Methods

Cell Isolation and Culture

Donor blood was acquired by the Johns Hopkins Hemapheresis Center operating under an institutionally approved protocol. PBMCs were isolated over a Ficoll-Hypaque gradient and viability was verified by trypan blue exclusion. Ten million cells/ml were cultured overnight in RPMI-1640 supplemented with 30% autologous human serum at 37°C and 5% CO_2. The next day, the cells were washed with phosphate-buffered saline (pH 7.2)(PBS) and adherent cells were removed with gentle scraping. The adherent monocytes ($CD14^+$) were 70.59 ± 18.01% pure as determined by flow cytometry. To

increase purity, the B cells, T cells, and granulocytes were removed using magnetic bead separation (Miltenyi Biotech, Auburn, CA), which gave 86.30 ± 7.30% pure $CD14^+$ cells.

The enriched $CD14^+$ cells were cultured at a concentration of $5x10^5$ cells/ml in RPMI-1640 media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 2 mM HEPES, 100 μM non-essential amino acids, 100 units/mL penicillin, 100 μg/ml streptomycin (Gibco, Grand Island, NY), 1000 U/ml of interleukin-4 (IL-4), and 50 ng/ml of granulocyte-macrophage colony stimulating factor (GM-CSF) (BD Biosciences, San Diego, CA). The cells were cultured at 37°C in 5% CO_2. The media and cytokines were replaced every two days. On day 6 the cells were treated with 200 ng/ml lipopolysaccharide (LPS, Sigma-Aldrich, St. Louis, MO) or infected at a multiplicity of infection (MOI) of 5 with the Chicago-1 strain of MV and incubated for 1 hour at 37°C in 5% CO_2. The cells were then washed and resuspended in fresh media and cytokines.

Virus Preparation

Chicago-1 strain MV was used to infect vero cells at .005 MOI. After 4 days of culture the cells were frozen at –80°C, thawed, and cellular debris was removed by centrifugation. Virus was concentrated by spinning at 80,000 x g for 1 hour at 4°C. Aliquots of these stocks were grown in antibiotic-free media for 14 days to verify lack of contamination.

Flow Cytometry

Cells were blocked with 10% donor human serum in PBS for 20 minutes, washed, and stained for 1 hour on ice using a panel of monoclonal antibodies including mouse anti-human CD80, CD86, CD83, HLA-DR, CD1a, CD14 and the appropriate isotype

controls (BD Biosciences). Analysis was performed using a FACScalibur flow cytometer and CellQuest software (BD Biosciences).

RNA Isolation and cDNA Synthesis

Total RNA was isolated from control cells and at 3, 6, 12, and 24 hours post-infection using the RNeasy Kit (Qiagen, Valencia, CA) and following the manufacturer's protocol. Gene array analysis was performed on Affymetrix GeneChip® U95Av2 (Affymetrix, Santa Clara, CA) following the manufacturer's protocol. Briefly, 15 μg of RNA was used to synthesize cDNA in two steps using the Superscript Choice System (GibcoBRL, Rockville, MD) and the reverse transcription primer T7-$(dt)_{24}$, [5'GGCCAGTGAATTGTAATACGACTCACTATAGGGAGG CGG$(T)_{24}$] (GENESET Corp., La Jolla, CA). For one set of timecourse data (0, 3, 6, 12, and 24 hours), an equal amount of RNA from two separate experiments was pooled to yield sufficient RNA for gene array analysis. Following cDNA synthesis, the product was purified using phase lock gels (Brinkmann Instruments, Westbury, NY).

cRNA Synthesis

The cDNA product was used to synthesize biotin-labeled cRNA with the BioArray™ High Yield™ RNA Transcript Labeling Kit (Enzo Diagnostics, Inc., Farmingdale, NY) following the manufacturer's protocol. After the *in vitro* transcription reaction, unincorporated nucleotides were removed using the RNeasy Kit. The cRNA was then precipitated, washed twice with 80% ethanol, dried, and resuspended in RNAse-free water. The RNA was quantified and 20 μg were fragmented by metal-induced hydrolysis. The products were verified by gel electrophoresis.

Hybridization, Staining, and Scanning

The fragmented cRNA was hybridized to the human U95Av2 GeneChip® array (Affymetrix) along with 50 pM control oligo B2 (5' bioGTCAAGATGCTACCGTTCAG 3') (Affymetrix), with the control RNA of Bio B (150 pM), Bio C (500 pM), Bio D (2.5 nM), and Cre X (10 mM) (Affymetrix), and 0.1 mg/ml herring sperm DNA (Promega, Madison, WI). Hybridization was done at 42ºC and 60 rpm in a hybridization oven (Affymetrix) for 16 hours. The chips were washed using the Affymetrix fluidics protocol EukWSH-4 and stained with streptavidin-phycoerythrin (Molecular Probes, Inc., Eugene OR) at a final concentration of 10 μg/ml in 6x SSPE buffer containing 1 mg/ml acetylated bovine serum albumin (Sigma-Aldrich). The chips were washed twice and scanned with the HP GeneArray Scanner (Hewlett-Packard, Santa Clara, CA).

Normalization and RMA

The affy package, developed by Irizarry et al. as an add-on to the software package R, was used to analyze the gene array data[15]. MVA plots revealed that the greatest distortion occurred at the lowest expression levels (data not shown). Background correction, normalization and summarization were done using quantile normalization and robust multiarray analysis (RMA). The processed data set is available at the GEO website, accession number GSE980 (www.ncbi.nlm.nih.gov/geo/).

SAM

Significance was determined using Significance Analysis of Microarrays (SAM)[16]. For this analysis, four control and three experimental samples were compared using the two-class, unpaired algorithm at two timepoints, 6 and 24 hours. The significantly regulated genes identified at each timepoint were combined. Only genes with a fold change of 2.0 or more were considered for significance. A false discovery

rate (FDR) under 10% at the 90th percentile was chosen. The results are shown in Supplementary Table 2.1. A higher threshold, with an FDR under 1% at the 90th percentile, was also examined and yielded similar conclusions (data not shown).

Comparisons

Comparisons were made to three other published experiments: 1) Huang et al. treated DCs with *Candida albicans*, mannose, *Escherichia coli*, LPS, influenza virus, and double-stranded RNA (polyinosine-polycytidine, PIC), over 36 hours, 2) Vahey et al. treated PBMCs with HIV over 72 hours, and 3) Browne et al. treated human foreskin fibroblasts with human cytomegalovirus (HCMV) over 48 hours[17, 18, 19]. The Huang data is available at http://web.wi.mit.edu/hacohen/dc, while the Vahey and Browne data are available from the GEO website, accession numbers GSE511 and GSE675, respectively. The results from these studies were handled the same as the MV data when possible, however the raw data from Huang et al. and Vahey et al. was not available so the background subtraction, normalization and summarization procedures were different. The SAM results for *C. albicans*, *E. coli*, and influenza virus from the Huang et al. study are shown in Supplementary Table 2.1. The LPS, mannose, and PIC samples could not be tested because they were only done in duplicates. No significantly regulated genes were found in the Vahey et al. study and the Browne et al. study did not have replicates so significantly regulated genes could not be determined.

Gene Annotation

The significantly regulated genes were annotated using Database Referencing of Array Genes Online (DRAGON) to assign SWISS-PROT keywords, SWISS-PROT subcellular localizations, and KEGG pathways[20, 21, 22, 23]. None of the classifications were

exclusive. An enrichment score was calculated for the SWISS-PROT keyword annotations where the enrichment score for a given keyword=((Significantly regulated genes with keyword)/(Total genes with keyword))/((Total significantly regulated genes))/(Total genes on chip))[24].

Graphing

Replicate measurements at each timepoint were averaged and divided by the time 0 mean to get the fold change. Duplicate genes were averaged. Probes from the HuGeneFL chip at times overlapped two or more probes on the U95Av2 chip. In these cases, the U95Av2 probe with the highest % similarity to the probe on the HuGeneFL chip was graphed. The graphs show the average fold change (except for HCMV, where the lines connect the actual measurements). Individual points represent the actual gene expression measurements. The DC controls and duplicate genes could have up to 15 measurements at each timepoint, but only three randomly chosen measurements were displayed. The PBMC control and HIV samples were normalized such that they could contain negative values, which are undefined on the log scale and were not plotted.

Results

Dendritic Cell Maturation

PBMCs were isolated from leukopheresis products and cultured with GM-CSF and IL-4 for six days. The cells showed the expected immature DC surface marker phenotype: $CD14^{-}$, $CD1a^{+}$, $CD83^{-}$, $CD80^{low}$, $CD86^{low}$ and $HLA\text{-}DR^{low}$ (Supplemental Figure 2.1). These immature monocyte-derived DCs were treated with 5 MOI of Chicago-1 strain MV or 200 ng/ml of LPS. In agreement with previous results, flow

cytometric analysis at 24 hours post-infection revealed that the cells had matured in response to the stimulation by down regulating CD1a and up regulating CD83, CD80, CD86 and HLA-DR in a manner similar to cells stimulated with LPS (Supplemental Figure 2.1)[7, 25]. The mRNA expression changes for these molecules, derived from the gene array experiments (see below), concur with the flow cytometry results, except for HLA-DR, which is known to be stored intracellularly in immature DCs and is transported to the plasma membrane following maturation (Supplemental Figure 2.2)[26]. (Similar results from the comparison experiments are shown in Supplementary Figure 2.2. See below). Total RNA was isolated and used for gene array analysis and RT-PCR at 0, 3, 6, 12, and 24 hours post-infection.

Gene Array Analysis

RNA from control and MV-infected DCs was isolated and used in gene array analysis using the Affymetrix GeneChip® system. Normalized expression values were used in Significance Analysis of Microarrays (SAM). We limited the false discovery rate to 10% at the 90th percentile and a fold-change > 2.0 for determining significance. In the MV-infected DCs there were 622 significantly up regulated genes and 931 down regulated genes. We compared our results to dendritic cells treated with other pathogens and found 47 up regulated genes in *C. albicans*-treated DCs, 106 up regulated genes in *E. coli*-treated DCs, and 72 up regulated genes in influenza-treated DCs. There were very few down regulated genes found in these studies, so they were not analyzed further. A summary of the significantly regulated gene results is shown in Supplementary Table 2.1. The complete significantly regulated gene results are available in Supplementary Tables 2.2-2.5.

Gene Classification

The significantly regulated genes were annotated with SWISS-PROT keywords using DRAGON. Only groups with enrichment scores >2.0 that contained at least 10 genes from the measles data (or 5 from the Huang et al. data) were analyzed further. The results for the up regulated and down regulated keyword groups are shown in Tables 2.1 and 2.2, respectively. Approximately 20% of the genes could not be classified or were unknown using this method. The complete keyword results are available in Supplemental Tables 2.6 and 2.7. The most up regulated classifications included anticipated results such as interferon induction, antiviral, inflammatory response, cytokine, chemotaxis, and SH2 domain (Table 2.1). Genes from these groups overlap with the prenylation, apoptosis, growth factor, signal anchor, and zinc groups. All of these classifications were up regulated in each DC sample. There were some surprising results as well, such as the metal-thiolate cluster classification induced by all of the pathogens and the chromosomal protein classification largely induced by measles infection.

The different statistical procedures used made it difficult to compare the 11 experiments in a systematic way, so it was important to examine the gene expression changes individually. Note that in the graphs all of the genes in a particular group are shown for completeness, but this does not mean they were significantly regulated. The significantly regulated genes found in each grouping are provided in Supplemental Tables 2.8-2.12.

The interferon-induced group was composed of genes for well-known IFN-induced proteins like chemokines, 2’5’-oligoadenylate synthetase (OAS), Mx, interferon-

regulatory factors (IRF), interferon-induced genes (IFI) and proteasomal components (Sup Figures 2.3-2.14). Nearly all of the treatments induced the OAS, Mx, IRF1, and IRF7 genes. In addition, gene names containing "interferon-induced" were strongly up regulated in DCs, but less so in PBMCs and fibroblasts. Interestingly, dsRNA-dependent protein kinase (PKR) was induced by every treatment except measles (Figure 2.1). This result was verified by RT-PCR. The chemokines CCL5, CCL8, CCL19, CXCL2, CXCL10 and CXCL11 were up regulated by all the DC treatments. CCL1, CCL2, CCL7, CCL15, CCL20, CXCL3, and CXCL9 were differentially regulated between treatment groups. Another DC maturation marker, CCR7, was strongly induced in DCs by all treatments at the earliest timepoints, except for PIC where it was not induced until 24 hours post-treatment.

The second induced group had the keyword antiviral and was composed of the interferon and Mx genes. Measles virus induced all of the interferon-α genes except IFNA8, while influenza virus induced IFNA1, IFNA2 and possibly others, but more weakly than measles (Figure 2.2). None of the other treatments induced interferon-α genes. Interferon-β and interferon-ω were strongly induced by measles and interferon-γ was weakly induced, while the other pathogens had unique patterns (Figure 2.3). Biologically active interferon from MV-infected DCs was measured with a cellular protection assay, which found that it increased through 12 hours and maintained that level through 24 hours (Figure 2.4).

The third classification group was metal-thiolate cluster, which was composed of genes from the metallothionein family (Figure 2.5). Genes in this family are responsible for binding trace metals, i.e. zinc, and have not been well described in the immune

response. Metallothioneins-1G and -1H are potently induced in all of the DC systems, but not in PBMCs or fibroblasts. Five of the seven MT1 genes were significantly regulated in measles virus-infected DCs, but not as strongly as in the other systems.

The inflammatory response group was mostly chemokines, while the cytokine group was composed of chemokines, colony-stimulating factors (CSF), interferons, interleukins, and members of the TNF superfamily (TNFSF, Sup Figures 2.15-2.18). Measles significantly up regulated a variety of interleukins, including IL-1β, -6, -8, -11, -12A, -12B, and –15. Measles also induced the most TNF superfamily members, with members 1, 2, 4, 7, 8, and 10. The other DC samples induced TNFSF1 and TNFSF10 while HCMV uniquely induced TNFSF9 and TNFSF10.

The chromosomal protein group was mostly composed of histone 1, H2b and H1 family members (Sup Figures 2.19-2.22). Many histone genes are increasing in the other treatments, but were not significantly regulated, possibly due to their low fold change values and large variability.

The down regulated classifications fall into five general groups (Table 2.2). The first group contains the highly connected ubiquinone, hydrogen ion transport, inner membrane, NAD, mitochondrion, transit peptide, and oxidoreductase groups. The genes in these classifications encode the proteins that form the five complexes of the oxidative phosphorylation machinery: NADH dehydrogenase, succinate-Q reductase, ubiquinol-cytochrome c reductase, cytochrome c oxidase, and ATP synthase (Sup Figures 2.23-2.27). The second group includes the ribosomal proteins and the largely overlapping classifications initiation factor, protein biosynthesis, and helicase. These groups contain

both nuclear and mitochondrial ribosomal proteins and the eukaryotic translation initiation factor family members (Sup Figures 2.28-2.33).

The third group is the chromatin regulators, which are composed of histone deacetylase genes and chromobox homologs (Sup Figures 2.34-2.35). The last two groups, lysosome and DNA repair, had a mixture of genes but no readily identifiable gene groups.

Subcellular Localization and Pathway Identification

The significant genes were also annotated and grouped according to their SWISS-PROT subcellular localization. Table 2.3 shows that most locations had the same relative ratio of up-regulated to down-regulated genes (≈0.7), except for the secreted, microsomal and type II membrane proteins, which were induced, while the mitochondrial and lysosomal/endosomal localizations were depressed. These results support the classifications shown in Tables 2.1 and 2.2, where the dendritic cells are beginning to secrete a large number of immunomodulatory factors while the mitochondrial respiratory chain and lysosome are shutting down. This classification procedure yielded new biological insights also, such as insulin-like growth factor binding proteins (IGFBP) from the secreted group. Measles, influenza, and *E. coli*-infected DCs secreted IGFBPs 4 and 6 while HIV-infected PBMCs produced IGFBP2 (Sup Figure 2.36).

A final way to look at the results is to use the KEGG database, which groups genes according to pathways or protein complexes. Only those pathways that had at least ten regulated genes are shown in Table 2.4. Three immune signaling pathways were induced: MAPK, Toll-like receptor, and Jak-STAT signaling pathways. The Toll-like receptor pathway was increased by all of the pathogens, while the Jak-STAT pathway

was increased by measles, *C. albicans* and *E. coli*, and the MAPK pathway was up-regulated by measles and *E. coli* (Sup Figures 2.37-2.43).

The KEGG classification method was the only one to highlight the interleukin, chemokine, and TNF receptors (Sup Figures 2.44-2.47). Again, the DC experiments showed differences with the PBMCs and fibroblasts, with IL2RA, IL7R, IL10RA, and IL15RA mRNAs being induced in most of the DC samples while IL1R2 was down regulated. The final up regulated pathways, apoptosis and calcium signaling, included genes from the NF-κB family (Sup Figure 2.48).

The most down regulated pathway was oxidative phosphorylation, which overlapped with ATP synthesis, followed by ribosomes. The cell cycle, focal adhesion, and regulation of actin cytoskeleton were composed of a diverse array of genes. The down regulated genes from the calcium signaling pathway included ATPases, adenylate cyclases and solute carrier family members, which were seen in the keyword classifications and the guanine nucleotide binding proteins, which were new (Sup Figures 2.49-2.59).

Discussion

We have analyzed the gene expression changes of dendritic cells infected with MV over the course of 24 hours and compared the results to other pathogen-host cell systems. We have generated a list of 1,553 significantly regulated genes that increase or decrease by 2-fold or more after exposure of immature, monocyte-derived DCs to MV. With this large dataset we were able to identify cellular pathways and compartments that are changing significantly following infection.

Many of the up regulated genes coded for known antiviral or interferon-induced proteins, but unexpected observations included genes in the histone and metallothionein families. The cellular antiviral response to MV has not been extensively characterized, with a few studies describing the interferons, OAS, IRF, and Mx genes[27, 28, 29, 30, 31, 32, 33, 34, 35]. Measles virus induces IFN-α/β and IL-6 in lung epithelial cells, consistent with our findings in DCs[36]. Microarrays were also used to study measles virus infection of PBMCs at 48 hours[37]. Of the ten induced genes identified in their study, six were on the U95Av2 chip and 5 of those, NF-κB p52, IRF7, 2'5'-oligoadenylate synthetase, IFN-α and IFN-β were significantly up regulated in our study.

Interestingly, although most treatments did not induce interferons to a large extent, interferon-induced genes were strongly induced, suggesting there may be an alternative signaling mechanism for the induction of these genes. Because the interferon-α results for measles virus are so uniform it is difficult to conclude that the probes are independently measuring the different interferon-α genes, although they seemed to act more independently in the influenza system. Nevertheless, the results show that microarrays, with independent confirmation, could be a powerful tool to study the interferon system in different virus-host cell systems to determine when the different interferon-α genes are expressed.

This study also found a number of new genes involved in the innate immune response to pathogens such as members of the histone, histone deacetylase, chromobox homolog, metallothionein, and IGFBP families. In addition, IL-11, IL-15 and a number of TNF superfamily members have not been studied in the context of measles virus

infection. These are only the characterized genes, and the microarray data identified 321 unclassified genes significantly regulated during the response (Sup Table 2.2).

It has long been observed that host cell transcription and translation are altered during viral infection[38]. This is being confirmed by numerous gene array studies, which often find more down regulated genes than up regulated genes following viral infection. In addition to our study, this has also been described in herpesvirus-infected endothelial cells, vaccinia virus- and influenza virus-infected HeLa cells, HIV-infected $CD4^+$ T cells, and human cytomegalovirus-infected fibroblasts[39, 40, 41, 42, 43]. This down-regulation is probably due to replicating virus, as Browne et al. showed that UV-inactivated virus down regulated significantly fewer genes than the live virus[19]. Few down regulated genes were found in the comparison data examined in this study, probably because the normalization procedures used failed to reduce the signal-to-noise ratio, making it difficult to detect significantly regulated genes at low expression levels.

Two large gene groups were down regulated following MV infection: oxidative phosphorylation and protein biosynthesis. Although the shutdown of host protein synthesis has been previously observed, the shutdown of oxidative phosphorylation is an unexplored area. Whether shutting down oxidative phosphorylation is an antiviral response, part of the apoptotic process, or has some other role in the virus-host relationship is quite unclear. Genes in the lysosome, DNA repair, cell cycle, focal adhesion and regulation of actin cytoskeleton classifications did not fall into any clear groups. These classifications may represent emergent properties, because the genes would not normally be grouped together except for their role in a particular biological process. The down regulation of lysosomal genes supports the hypothesis that DCs, upon

maturation, switch from a highly phagocytic, antigen-processing cell to a potent antigen-presenting cell[44]. The role the genes present in other classifications are playing in the immune response is not clear.

Currently, the varying array platforms, analysis algorithms, and annotation schemes make it difficult for laboratories to compare results. In addition, experimental differences, such as the treatment dose and duration, complicate analysis further. Despite these limitations, we found that it is possible to compare results from different systems and obtain meaningful results. These findings also suggest that the use of standard experimental designs and statistical procedures will increase the resolution of microarray data comparisons, yielding new insights into cell biology.

Acknowledgments

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Table 2.1 Significantly up regulated genes classified by SWISS-PROT keyword.

	Measles			*C. albicans*		*E. coli*		Influenza	
SWISS-PROT Keyword	Up	Down	ES	Up	ES	Up	ES	Up	ES
Interferon Induction	29	1	12.9	12	49.5	13	22.7	18	52.7
Antiviral	11	0	10.1	1	8.7	1	3.7	3	18.6
Metal-thiolate Cluster	6	0	8.7	2	21.2	5	22.5	2	15.0
Inflammatory Response	17	6	5.8	6	22.9	11	17.8	5	13.5
Cytokine	45	5	5.7	10	13.3	17	9.6	11	10.3
Chemotaxis	16	4	5.2	4	11.9	9	11.3	4	8.4
Chromosomal Protein	13	3	3.6	0	0	1	1.0	1	1.6
Prenylation	17	12	2.7	1	2.1	1	0.9	1	1.5
Apoptosis	29	11	2.5	2	2.3	7	3.4	2	1.6
Growth Factor	16	5	2.3	2	3.2	5	3.3	3	3.4
SH2 Domain	14	13	2.3	1	2.0	1	0.9	2	2.9
Signal-anchor	16	10	1.5	2	2.4	3	1.5	5	4.2
Zinc	30	26	1.3	4	2.4	11	2.8	4	1.7

Table 2.2 Significantly down regulated genes classified by SWISS-PROT keyword.

		Measles	
SWISS-PROT Keyword	Up	Down	Enrichment Score
Ubiquinone	0	13	6.4
Ribosomal Protein	0	36	4.3
Hydrogen Ion Transport	0	13	3.5
Inner Membrane	0	15	3.5
Chromatin Regulator	2	12	3.4
Initiation Factor	1	12	3.3
NAD	3	32	3.1
Mitochondrion	11	82	2.7
Transit Peptide	6	58	2.7
Protein Biosynthesis	3	17	2.5
Oxidoreductase	14	66	2.3
Lysosome	1	14	2.2
DNA Repair	1	17	2.1
Helicase	0	13	2.0

Table 2.3 Significantly regulated genes classified by SWISS-PROT subcellular localization.

SWISS-PROT	Measles		*C. albicans*	*E. coli*	Influenza
Subcellular Localization	Up	Down	Up	Up	Up
Unclassified	266	403	15	39	31
Membrane					
Integral	38	72	4	10	6
Type I	36	53	3	7	1
Type II	18	10	2	3	5
Miscellaneous	20	27	0	0	0
Nuclear	104	167	6	9	8
Cytoplasmic	82	148	7	13	6
Mitochondrial	16	75	2	2	2
Secreted	78	25	13	23	13
Golgi/TGN	4	10	0	0	0
Endoplasmic Reticulum	8	14	0	0	0
Lysosomal/Endosomal	1	27	0	0	0
Peroxisomal	4	5	0	0	0
Extracellular	2	3	0	0	0
Microsomal	5	4	0	3	1

Table 2.4 Significantly regulated genes classified by KEGG pathway.

KEGG Pathway	Measles Up	Measles Down	*C. albicans* Up	*E. coli* Up	Influenza Up
71 Fatty Acid Metabolism	3	10	0	0	0
190 Oxidative Phosphorylation	0	33	0	0	0
193 ATP Synthesis	0	11	0	0	0
3010 Ribosome	0	21	0	0	0
4010 MAPK Signaling Pathway	26	19	0	3	0
4020 Calcium Signaling Pathway	11	17	0	3	0
4060 Cytokine-Cytokine Receptor Interaction	37	9	9	13	6
4110 Cell Cycle	7	13	0	1	2
4210 Apoptosis	19	7	1	2	1
4510 Focal Adhesion	12	17	0	1	0
4620 Toll-like Receptor Signaling Pathway	22	4	5	5	4
4630 Jak-STAT Signaling Pathway	21	4	3	6	0
4810 Regulation of Actin Cytoskeleton	4	17	0	1	0

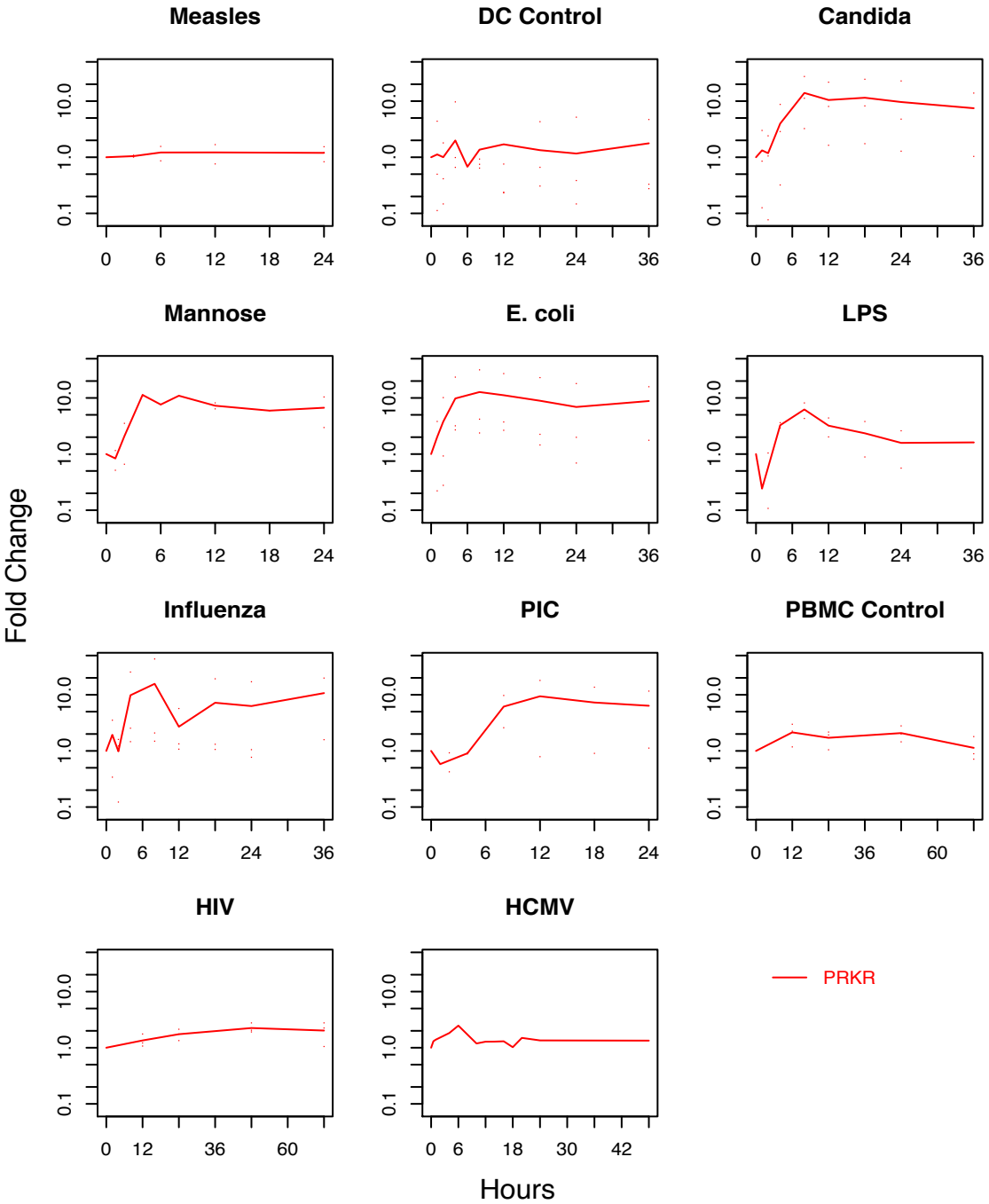

Figure 2.1 dsRNA-dependent protein kinase gene expression. Timecourse graphs of the PKR gene are shown for each of 11 conditions. DCs were treated with media, MV, *C. albicans, E. coli,* influenza, LPS, mannose or PIC. PBMCs were treated with media or HIV and fibroblasts were treated with HCMV. RNA was collected at various timepoints and expression levels were measured by microarray analysis. The lines show the mean fold change, normalized to the time 0 timepoint, while the points plot the actual fold change measurements.

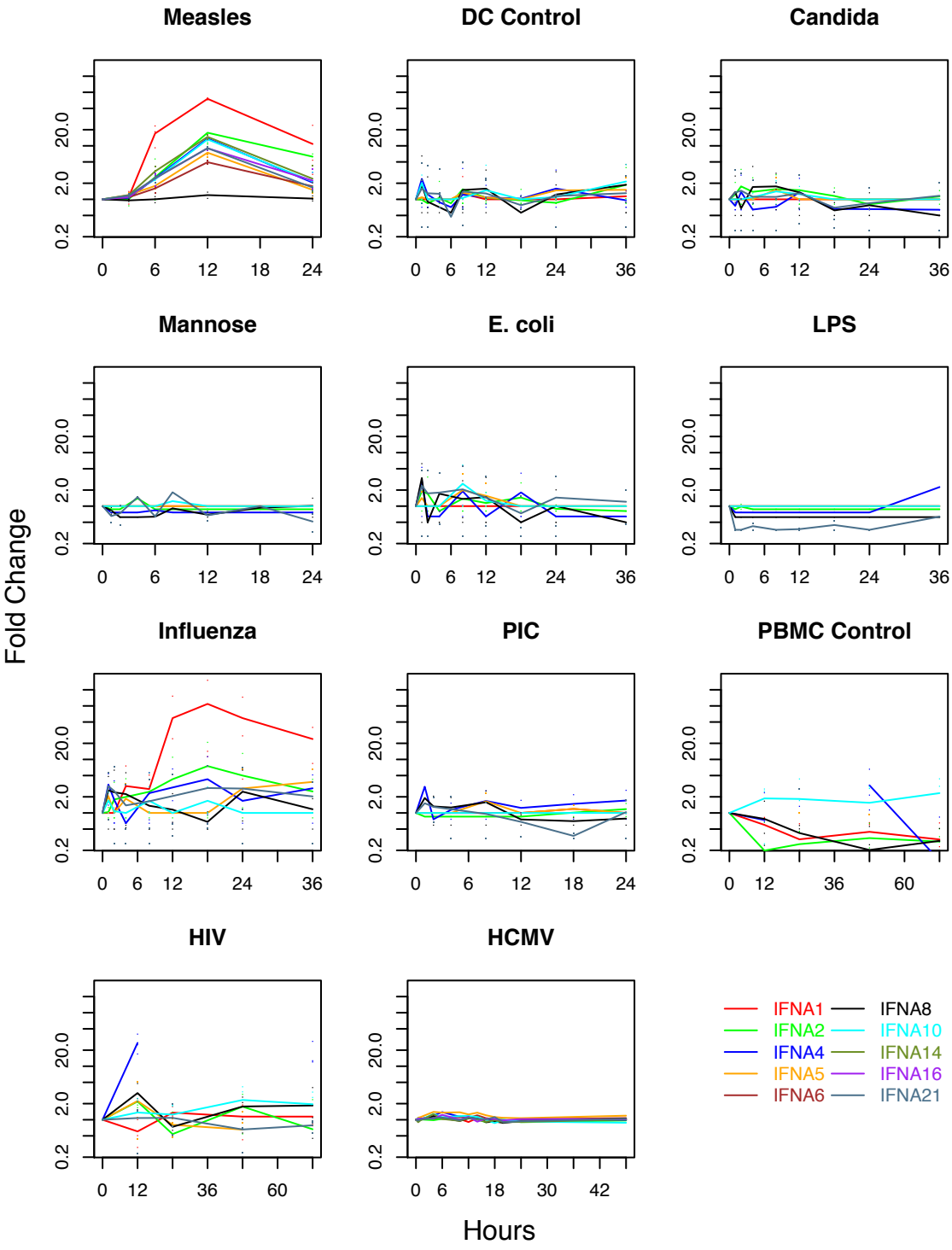

Figure 2.2 Interferon-α gene expression. Timecourse graphs for interferon-α genes in each of 11 conditions are shown as in Figure 2.1.

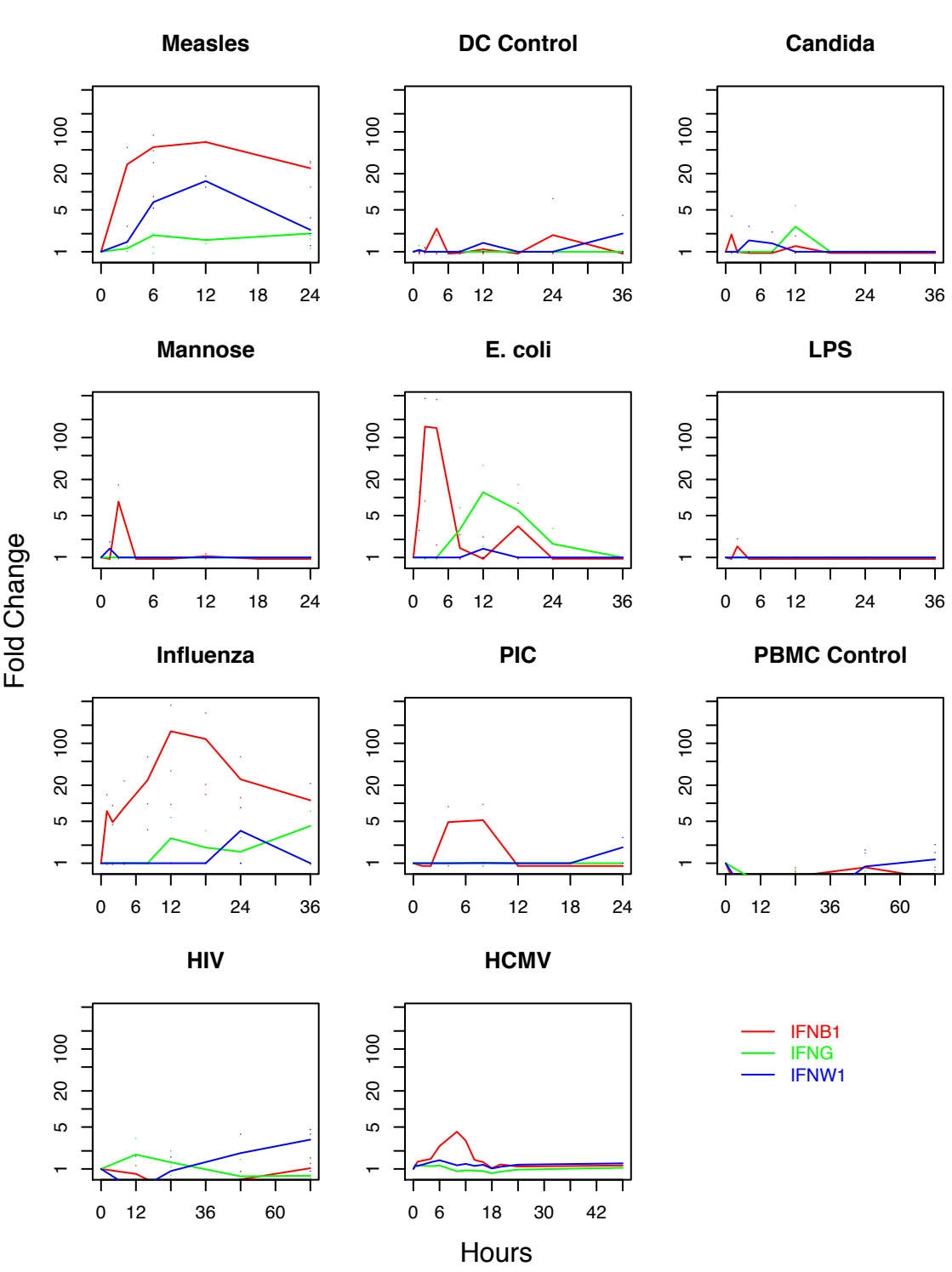

Figure 2.3 Interferon-β, -γ and -ω gene expression. Timecourse graphs for interferons-β, -γ and -ω in each of 11 conditions are shown as in Figure 2.1.

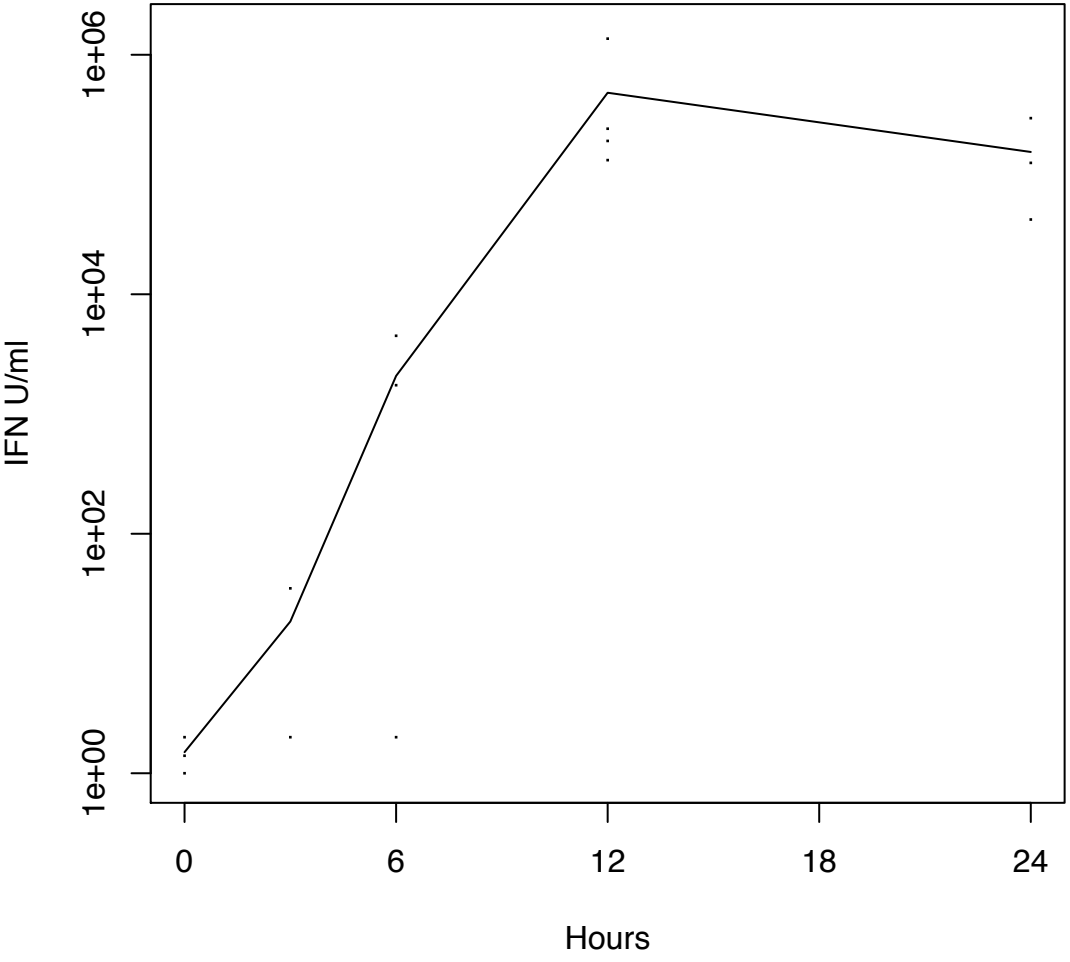

Figure 2.4 Production of biologically active IFN by MV-infected DCs. Points show the calculated IFN units per milliliter for the replicates and the line connects the means.

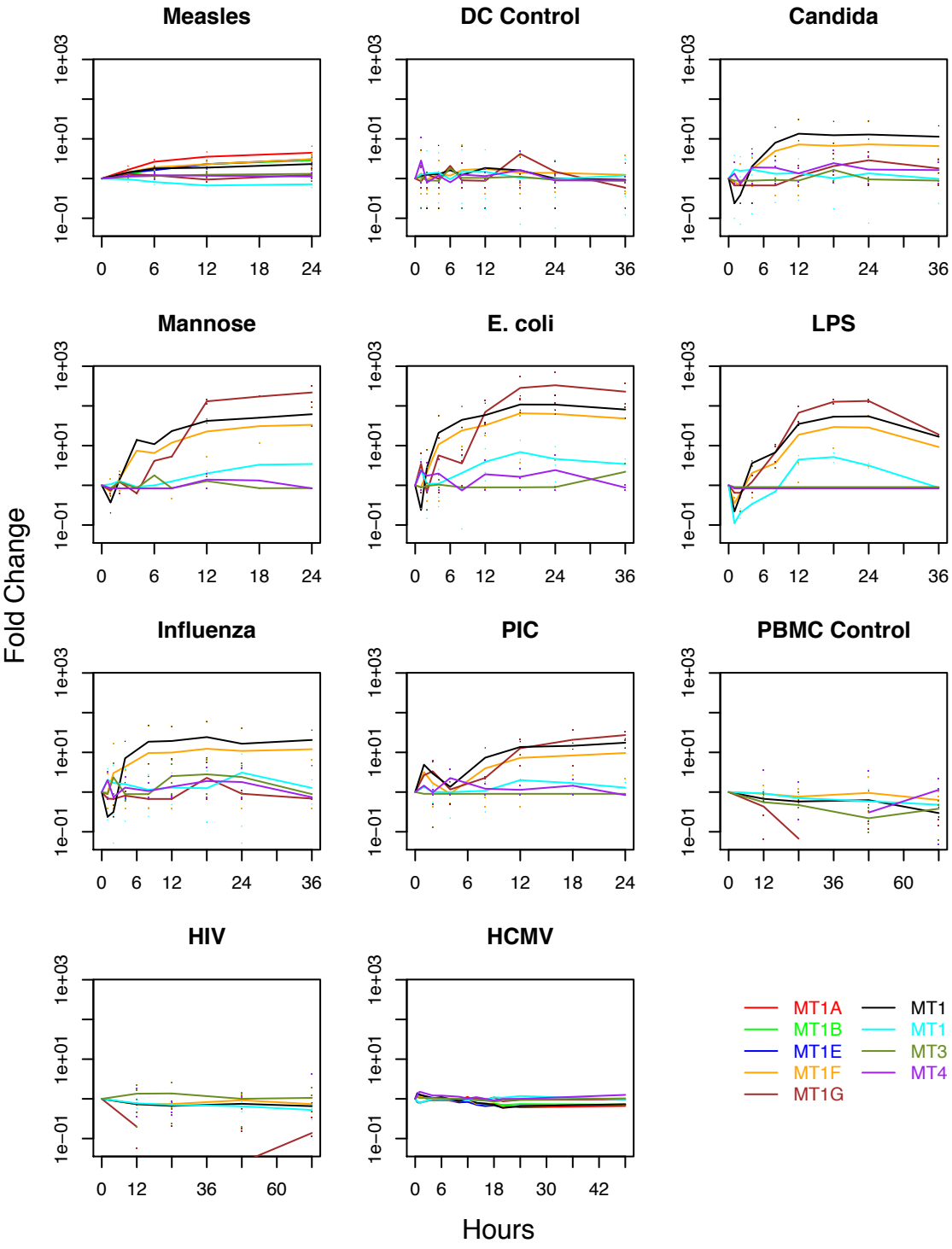

Figure 2.5 Metallothionein gene expression. Timecourse graphs for the metallothioneins in each of 11 conditions are shown as in Figure 2.1.

Chapter 3

Changes in PBMC Gene Expression During Acute Measles

Michael J. Zilliox, William J. Moss and Diane E. Griffin

Abstract

Measles virus continues to cause a great deal of morbidity and mortality despite the existence of a safe and efficacious vaccine. In this study we looked at the gene expression changes that occur during acute measles infection and convalescence in peripheral blood mononuclear cells (PBMCs). We found 13 significantly up regulated genes, including the cytokines interleukin-1β (IL-1β) and tumor necrosis factor (TNF) and the chemokines interleukin-8 (IL-8), CXCL2 and CCL4. There were 206 down regulated genes, which were mainly involved in three biological processes: transcription, signal transduction and the immune response. These results help understand the pathogenesis of acute measles virus infection.

Introduction

Measles virus is remarkable for the robust immune response it elicits in the face of a generalized immune suppression that lasts several weeks following acute infection. After infection of the respiratory epithelium, the virus is thought to travel to the regional lymph nodes via DCs or macrophages where it incubates for 10-14 days[1]. The virus replicates in lymphoid tissue and spreads throughout the body, triggering a robust immune response that starts with IgM antibody production to N at the time of the rash[2]. Eventually, antibodies are made to most MV proteins, with the predominant serotype being IgG1 followed by IgG4, IgG2 and IgG3[2, 3].

The CD8 T cell response is known to be important for viral clearance and immune memory. Patients with agammaglobulinemia develop the characteristic rash and recover from the disease, showing immunity to further exposure[4]. CD8+ T cells from normal patients have been shown to proliferate in response to MV-infected cells, which causes the characteristic rash[5, 6]. During the time of the rash there is significant evidence of immune activation including an increase in sCD4, sCD8, sIL-2R, neopterin (produced by activated macrophages), sFas, sFasL, and sTNFR[7, 8]. Every arm of the immune system is not activated though as NK cell activity is decreased during acute disease[9]. Lymphoproliferative responses to MV antigen and DTH responses are also severely repressed[1, 10].

A number of cytokines have been measured in the serum of infected patients during the immune response. IFNγ is detected early in the response and quickly returns to baseline shortly after the rash appears[11, 12, 13]. IL-2, IL-4, IL-13 and IL-10 were also elevated during acute infection with IL-4 and IL-10 persisting for several weeks

following viral clearance[11, 14]. IL-5 and IL-12 levels were lower than controls, while TNF and IL-1β are undetectable[11, 13, 15, 16]. These findings lead to the hypothesis that the early immune response is characterized by a Th1 type response followed by a prolonged Th2 response that lasts for several weeks and contributes to the immune suppression[17]. In this study, microarrays were used to globally survey mRNA levels in PBMCs to understand other immune changes that may be occurring during acute infection and convalescence.

Materials and Methods

Samples of peripheral blood were collected from children with measles at study entry, hospital discharge and 1-month follow-up in sterile tubes containing EDTA at the University Teaching Hospital in Lusaka, Zambia as previously described[18]. White blood cell counts and differential WBC counts were performed manually. IgM EIA confirmed measles infection and all children were negative for HIV infection. PBMCs from 5 children with measles and 3 controls were separated on Ficoll-Hypaque, washed and stored in RNAzol or RNA-Stat (Tel-Test, Inc., Friendswood, TX) at -80°C and at a later time RNA was isolated following the manufacturer's protocol. RNA quality assessment was determined by RNA Nano LabChip analysis on an Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA). All studies were done with protocols approved by both Johns Hopkins University and University Teaching Hospital.

Affymetrix GeneChip Protocols

For each sample, 100 ng of total RNA was processed according to the amplification protocol from the Affymetrix GeneChip Eukaryotic Small Sample Target

Labeling Technical Note, version 1 (Affymetrix, Santa Clara, CA). The protocol features two cycles of amplification, each containing a cDNA synthesis step followed by an *in vitro* transcription reaction to generate cRNA. The second IVT synthesis incorporates biotinylated ribonucleotides to produce labeled antisense cRNA targets for hybridization. Fifteen micrograms of cRNA were fragmented by metal-induced hydrolysis in fragmentation buffer (250mM Tris acetate pH 8.1, 150 mM MgOAc, 500mM KOAc) at 94^{o} C for 35 minutes. Hybridization cocktails were prepared as recommended for arrays of "Standard" format and applied to Affymetrix Human Genome U133A GeneChips. Hybridization was performed at 45^{o}C for 16 hours at 60 rpm in the Affymetrix rotisserie hybridization oven. The signal amplification protocol for washing and staining of eukaryotic targets was performed in an automated fluidics station (Affymetrix FS450) as described in the Affymetrix Technical Manual, Revision Three. The arrays were then transferred to the GCS3000 laser scanner (Affymetrix) and scanned at an emission wavelength of 570nm at 2.5 mm resolution. Intensity of hybridization for each probe pair was computed by GCOS 1.1 software.

Exploratory data analysis was performed on the samples using R v2.2.0 and the add-on package affy v1.5.8[19, 20]. Background correction, normalization, and summarization were performed using Robust Multi-Array Analysis[20]. Significance testing was performed with Significance Analysis of Microarrays (SAM) v2.21 on controls versus each of measles entry, discharge, and follow-up[21]. A gene was considered significant if it had a fold change >2.0 and a false discovery rate (FDR) <15% at the 90th percentile. There were no significantly regulated genes at entry, 224 at discharge, and 32 at follow-up. The results were pooled and duplicate genes were

averaged, leaving 13 up regulated genes and 206 down regulated genes. The genes were then annotated with the June 20, 2005 Affymetrix release and grouped into gene ontologies with Onto-Express using the default settings[22]. Only groups with a corrected p-value < 0.05 and including 5 or more genes were considered in the analysis. Genes were also annotated with Swiss-Prot keywords, subcellular localizations and KEGG pathways using Database Referencing of Arrays ONline (DRAGON)[23].

Results

Children with acute measles were admitted to the University Teaching Hospital in Lusaka, Zambia, where blood was collected following an institutionally approved protocol. Table 3.1 shows the clinical characteristics of the subjects. Both boys and girls were included ranging in age from 9 months to 79 months. Collected samples were calibrated by the number of days since the individual first developed a measles rash, which is typically 12-17 days after infection[1]. The median number of days post-rash at entry, discharge and follow-up were 4, 8 and 40, respectively.

The patient WBC counts, % lymphocyte, % CD4+ and % CD8+ T cells closely resemble those reported for the larger study from which these samples were drawn (Figure 3.1) [18]. The WBC counts are slightly elevated at discharge and follow-up with a lower lymphocyte percentage that increased to control levels by discharge. The CD4 percentages were lower at entry than control as has been reported, although they returned to baseline by follow-up in the larger study. These patients have slightly lower % CD4 at follow-up than the cohort average. The CD8 percentages were also very similar, although the controls in this study were on the low end of the larger control group.

RNA extracted from PBMC at each timepoint was run on microarrays. There were 13 significantly up regulated genes and 206 significantly down regulated genes. The complete results are shown in Supplemental Table 3.1. Significant genes were annotated with Onto-Express and DRAGON. The significant groups for the gene ontology biological processes annotation are shown in Table 3.2. The only up regulated groups were signal transduction and apoptosis, which overlapped significantly. The genes found in these groups included the cytokines IL-1β and TNF and the chemokines CCL4, CXCL2, and IL-8 (Figure 3.2). The up regulated genes all peaked at discharge and declined at follow-up, but not down to the control level.

The down regulated classifications fell into three broad biological processes: transcription, signal transduction and the immune response (Table 3.2). Transcription had the largest number of genes and included four classifications: regulation of transcription, DNA dependent, transcription, regulation of transcription from RNA polymerase II promoter and nuclear mRNA splicing, via spliceosome. The largest gene groups in these classifications were zinc finger proteins and transcription factors. These genes were being down regulated at entry and most of them hit their lowest expression levels at discharge and began to recover by follow-up, though not back to the control level (Supplemental Figure 3.1).

The second biological process was signal transduction, which included nine receptor genes and eight kinases. The immune response classifications included IL-16, IL-4R, IL-6R, IL-7R, IL-27RA, TNFSF3, CCR2 and CCR7. The cytokine and chemokine receptors had the lowest expression at discharge and did not fully recover by follow-up (Figure 3.3).

The subcellular localization of the proteins encoded by the significant genes is shown in Supplemental Table 3.2. The number of up regulated secreted proteins and down regulated nuclear genes were found to be significant using OntoExpress. The KEGG pathway, Swiss-Prot keyword and molecular function annotations supported the biological process results (Supplemental Tables 3.3-3.6).

Discussion

This study examined the gene expression changes in PBMCs from children infected with MV at hospital entry, discharge, and at 1-month follow-up. There were 13 up regulated genes and 206 down regulated genes. A number of the up regulated genes identified in this study have been examined in measles and other viral infections. IL-1β is a potent inflammatory cytokine produced by macrophages that is responsible for inducing fever and activating T cells and macrophages[24]. IL-1β mRNA is found up-regulated in MV-infected monocytes, glial cells, meningeal fibroblasts and a malignant glioma cell line[25, 26, 27]. Increased IL-1β protein was detected in the cerebrospinal fluid of SSPE patients and is produced by cultured PBMCs taken from patients during the convalescent phase of infection[28, 29].

TNF-α is another potent inflammatory cytokine and endothelial cell activator[24]. Its gene expression was also significantly up regulated in these patients, but this has not been consistently observed in other studies. Glial cells and malignant glioma cells have increased expression after MV infection, but meningeal fibroblasts and monocytes do not[25, 26, 27]. TNF-α protein levels are normal in the cerebrospinal fluid of SSPE patients and production by PBMCs is reduced during the acute phase of infection[28, 29].

Interestingly, TNF-α plasma levels and production by PBMCs from vaccinees is increased, suggesting there may be a difference between natural infection and vaccination in TNF-α production[30, 31]. ICAM-1 is expressed on lymphocytes, monocytes and neutrophils and is up regulated during inflammation[32]. It may serve as a costimulatory receptor for APC-T cell interactions and enhances antigen presentation[33]. It has been found to be higher in SSPE patients and following MV infection of astrocytes[28, 34].

There were several chemokines significantly regulated by measles virus, with CCL4 (MIP-1β), CXCL2 (GRO-β) and IL-8 being up regulated. CCL4 is produced and secreted by activated macrophages to attract other proinflammatory cells, such as CD4+ T cells or DCs to attract memory cells[35]. The chemokine has no effect on DCs and little on NK cells[36, 37, 38]. The only known receptor is CCR5, which is preferentially up regulated on Th1 cells and consequently, CCL4 has more activity for Th1 cells than Th2 cells[37, 39, 40]. It has also been shown that high amounts of CCL4 at the site of T cell activation will favor a Th1-type response[41].

CXCL2 and IL-8, which are structurally related, were also up regulated in response to measles[42]. The GRO family is produced by macrophages and serve as chemoattractants for neutrophils and basophils, but have little activity toward monocytes[42, 43, 44, 45, 46]. Previously, an increase in GRO-α (CXCL1) was observed during measles infection[47]. IL-8 is made by monocytes, T cells, neutrophils, fibroblasts, endothelial cells and epithelial cells to attract neutrophils, basophils and T cells[24, 37]. Both IL-8 and CXCL2 bind to CXCR2, but IL-8 also binds to CXCR1[37]. This may partially explain why IL-8 is more potent than CXCL2 for attracting neutrophils[45]. CXCL2 may contribute to lung injury during viral infection[44, 48]. The available IL-8 data

also supported our findings as the bronchial epithelial cell line, A549, produces IL-8 following MV infection and it is also increased in the plasma of infected monkeys 3 days post-infection[49].

The down regulated genes found in this study have not been well characterized in MV infection, although IL-16 plasma levels were measured in this cohort and were significantly down regulated at follow-up[50]. Microarray analysis showed that IL-16 was significantly down regulated at discharge and remained below the levels of control at follow-up. The discrepancy may reflect the time required to see a difference due to a change in gene regulation manifested in the serum.

There were two down regulated chemokine receptors, CCR2 and CCR7. CCR2 is highly expressed on monocytes, activated and memory T cells (both Th1 and Th2) and is down regulated when monocytes differentiate into macrophages[39, 41, 51]. It has two forms and both bind MCP1 (CCL2), 3 (CCL7) and 5 (CCL12), which are potent attractors of monocytes and T cells[24, 37]. Both TLR2 and TLR4 agonists down regulated CCR2 and measles H protein has been shown to activate TLR2[52, 53]. Consistent with our findings, *Candida*-treated monocytes down regulated CCR2[54].

CCR7 is expressed on naïve T cells, activated B and central memory T cells, but it is absent on effector memory cells[55, 56, 57]. CCR7- cells express receptors for migration to inflamed tissues and display immediate effector function, suggesting the down regulation seen during acute measles could be due to the ongoing T cell effector response[1, 58].

Several studies have looked at gene expression changes in PBMCs following infection or disease. Patients with autoimmune diseases showed distinct patterns

compared to acute measles infection[59]. PBMCs from SARS patients showed no up regulation of cytokines or other molecules involved in an adaptive immune response. Most of the genes up regulated were of the innate immune system[60]. Smallpox virus-infected monkeys showed up regulation of a number of genes involved in cell cycle regulation, as well as immune modulators[61]. SIV-infected macaques sampled at 3 and 7 weeks had two down regulated zinc finger proteins similar to measles, but otherwise showed quite different responses[62]. CXCL2 was up regulated in LPS challenged humans, but the down regulated genes were involved in respiration, protein synthesis and degradation[63]. Acute measles changes were most similar to those induced by HIV infection in humans, where signal transduction, immune response and transcription were also large down regulated groups[64].

Since the cell composition of the PBMC compartment is changing during this time (Figure 3.1), it is possible that the gene expression changes are due to alterations in the cell composition. However, since the largest alterations in cell composition occurred at entry and there were no significantly regulated genes at this time, this suggests that the significantly regulated genes at follow-up and discharge may reflect gene expression changes. By purifying blood cells and sampling more timepoints, gene expression analysis could contribute more understanding to measles virus pathogenesis.

Acknowledgements

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the statistical analysis. The expertise, facilities, and instrumentation for Affymetrix GeneChip experimentation are provided and supported by the Johns Hopkins University Malaria Research Institute.

Table 3.1 Clinical characteristics of patients and controls.

	Age (Months)	Sex	Days post-rash at: Entry	Discharge	Follow-up
Control 1	26	Female	NA	NA	NA
Control 2	15	Male	NA	NA	NA
Control 3	9	Male	NA	NA	NA
Patient 1	21	Male	3	8	61
Patient 2	79	Female	2	4	36
Patient 3	9	Male	7	11	41
Patient 4	11	Female	4	9	39
Patient 5	8	Male	4	6	40

NA=Not Applicable

Table 3.2 Significant genes grouped by GO biological process.

GO ID	Function Name	Unique Input	Corrected P-Value
	Up Regulated		
7165	Signal transduction	6	1.64E-05
6915	Apoptosis	5	2.49E-07
	Down Regulated		
6355	Regulation of transcription, DNA-dependent	35	.0019
6350	Transcription	30	.0012
7165	Signal transduction	24	.038
6468	Protein amino acid phosphorylation	13	.031
6955	Immune response	12	.0040
6357	Regulation of transcription from RNA polymerase II promoter	11	.0025
398	Nuclear mRNA splicing, via spliceosome	7	.018
7166	Cell surface receptor linked signal transduction	6	.019
19735	Antimicrobial humoral response (sensu Vertebrata)	6	.0023
6968	Cellular defense response	5	.0058

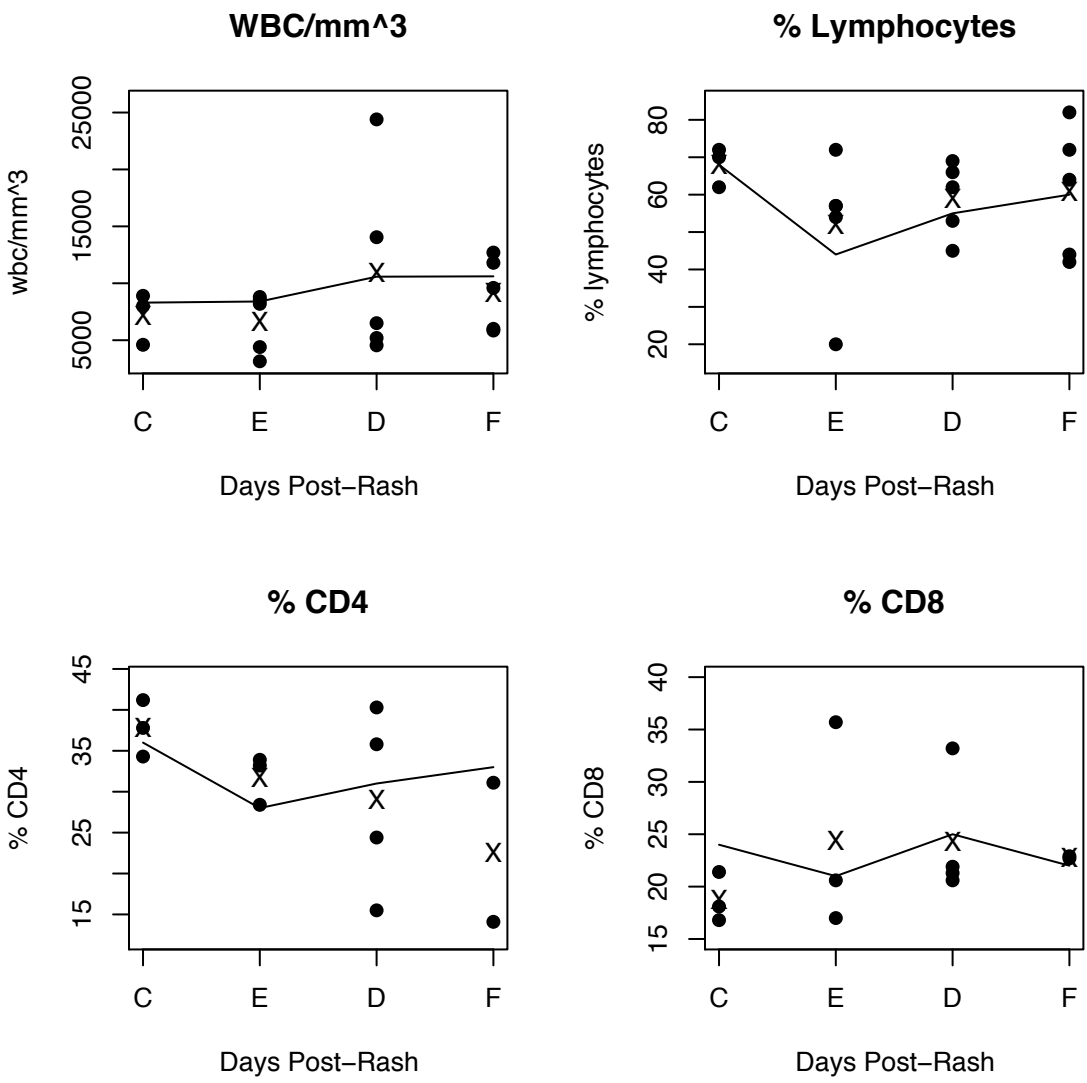

Figure 3.1 Blood lymphocyte counts for patients with acute measles virus infection. The individual counts are shown for each patient with filled circles while the median value is displayed with an "X". The days post-rash are shown by (c)ontrol, (e)ntry, (d)ischarge and (f)ollow-up, which correspond roughly to 0, 4, 8 and 40 days, respectively.

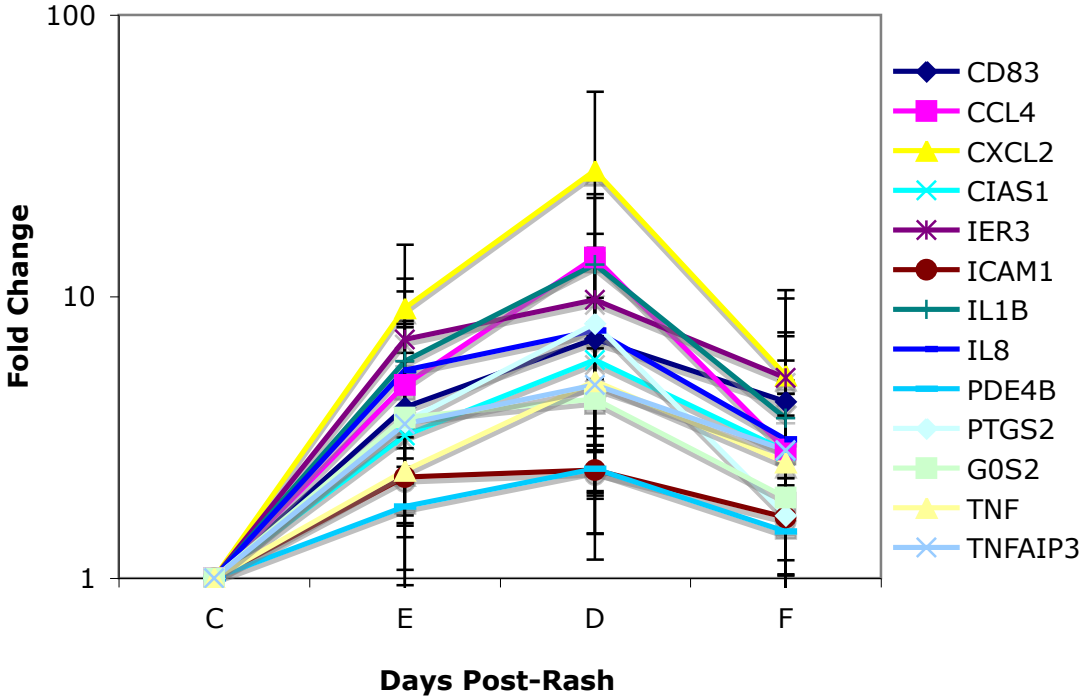

Figure 3.2 Up regulated genes. The mean and standard deviation of the fold change for each gene is shown. The days post-rash are shown as in Figure 3.1. CIAS1- cold autoinflammatory syndrome 1. IER3- immediate early response 3. ICAM1- intercellular adhesion molecule 1. IL- interleukin. PDE4B- phosphodiesterase 4B. PTGS2- prostaglandin-endoperoxide synthase 2. G0S2- putative lymphocyte G0/G1 switch gene. TNF- tumor necrosis factor. TNFAIP3- tumor necrosis factor alpha-induced protein 3.

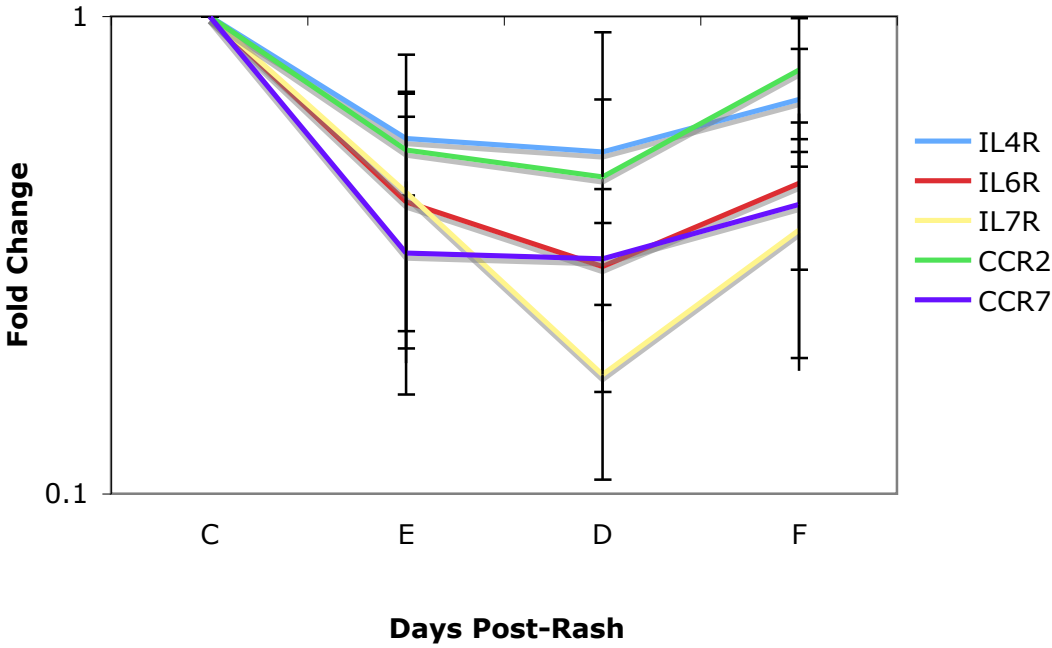

Figure 3.3 Examples of down regulated cytokine receptors and chemokine receptors. The mean and standard deviation of the fold changes for each receptor is shown. The days post-rash are shown as in Figure 3.1.

Chapter 4

Measles Virus Vaccination in BALB/c Mice

Michael J. Zilliox and Diane E. Griffin

Abstract

Measles vaccination has reduced morbidity and mortality for humans in many parts of the world, but there are still a significant number of deaths in large parts of Africa and Southeast Asia. Although a safe and efficacious vaccine is in widespread use, it has not completely penetrated these endemic areas because it is ineffective in infants less than 6 months of age due to protection from maternal antibody and the immaturity of the infant's immune system. As measles virus eradication is an elusive and important goal, it would be useful to design a vaccine that can be given earlier in life, preferably at birth. In this study we evaluated a new alphavirus replicon particle expressing the measles virus H protein (VCR-H) and the formalin-inactivated measles vaccine (FIMV) in mice. The results show that both vaccines induced significant antibody production, but only the VCR-H vaccine induced a robust interferon-γ response. Microarray analysis suggested molecular differences between the two vaccines and areas for further research.

Introduction

Enders and Peebles isolated the original vaccine strain of measles virus, Edmonston, in the 1950's using a monkey kidney cell line[1]. The strain was passaged on human renal and amnion cells and then in chick embryos to attenuate it[2]. This live attenuated vaccine (LAV) induced a robust immune response but it did have some mild side effects, which could be lessened by giving γ-globulin at the time of vaccination[3, 4]. To attenuate the vaccine further it was again passaged in chick embryos. These additional passages resulted in the Schwarz and Moraten vaccine strains, both of which had fewer side effects following vaccination than the Edmonston strain, but also had a lower seroconversion rate[5, 6]. The LAV induced seroconversion in 95% of individuals after one dose[7, 8, 9, 10, 11].

Another approach to vaccination for measles was to inactivate the virus with formalin and saturate it with alum. This formalin-inactivated measles vaccine (FIMV) was well tolerated, but it required three doses to achieve 90% seroconversion[7, 12, 13, 14]. In addition to the lower seroconversion rate, the antibody levels induced by the inactivated vaccine began dropping within a few months after vaccination, causing many individuals to quickly lose protection[7]. When some of these individuals were exposed to circulating virus, they developed an atypical disease that was first described by Rauh and Schmidt[15]. They found that 43.2% of children given the three doses of killed vaccine developed natural measles, modified measles or a more severe atypical disease within the next 2.5 years. Many other groups described atypical disease and found it could occur decades after the original vaccination, even if the individual had received the LAV in the

interim[16, 17, 18]. Other inactivated paramyxovirus vaccines have also been found to predispose to a more severe disease after natural infection[19, 20].

After studying individuals with atypical measles, it was found that the antibody profile in those people was different from those who received the LAV. Specifically, the LAV induced antibodies against both H and F while the killed vaccine only induced antibodies against H[21, 22, 23, 24, 25, 26]. It was postulated that the lack of these antibodies allowed the virus to spread from cell to cell so the vaccine was not protective, but H protein was continually produced which induced a hyperimmune response that caused the atypical disease[21, 26]. This hypothesis was never proven *in vivo* though.

In the 1990's, a monkey model of the atypical disease was developed which provided many insights. Monkeys given the FIMV had lower levels of IL-12, higher IL-4 and increased neutrophils[27]. The monkeys given FIMV did not develop a high-affinity antibody response, while the LAV monkeys did[28]. Experiments also showed that the atypical disease was the result of immune complex formation and increased eosinophilia[29].

Measles virus eradication is hindered by the immaturity of the infant immune system. Studies have shown that the responses in infants are qualitatively different from the responses in adults[30]. Passive antibodies from the mother also inhibit seroconversion to the vaccine, whether the mother has had wild measles infection or the vaccine herself[31]. A number of approaches have been taken to developing a new measles vaccine including using vesicular stomatitis virus, vaccinia virus and salmonella as vectors, administering the vaccine through aerosol or just producing a DNA or RNA vaccine

[32, 33, 34, 35, 36, 37, 38]. In this paper we compare the immune responses and gene expression patterns of mice immunized with a new alphavirus replicon particle vaccine and the formalin-inactivated vaccine.

Materials and Methods

Vaccinations and Cell Isolations

All mouse work was completed under an institutionally approved protocol. Female 5-6 week old BALB/c mice were vaccinated in the rear footpads with saline (30 μl + 40 mg/ml lactose), a previously described chimeric Sindbis virus-VEE replicon construct containing the complete coding sequence of the MV H protein (VCR-H, 5 x 10^5 IU particles/footpad) in saline with lactose or the formalin-inactivated measles vaccine (30 μl/footpad, FIMV, Pfizer, New York, New York)[29, 39]. Control and vaccinated mice were sacrificed on days 0, 4, 7, 10, 14, 21, 28, 35 and 42. Spleens, popliteal, superficial inguinal and axillary lymph nodes were removed. CD4+, CD8+ and CD19+ cellular subsets were enriched using magnetic beads by following the manufacturer's protocol (Miltenyi Biotec, Auburn, CA). For some assays, CD4+ T cells or CD8+ T cells were depleted using magnetic beads. On average, CD4+, CD8+ and CD19+ cells were isolated to 86.7 ±4.1%, 82.4±6.1% and 87.7±4.4% purity, respectively. Blood was collected, allowed to clot for 30 minutes at room temperature and centrifuged at 16,000 x g for 30 minutes to obtain the serum.

ELISA

The protocol was followed as previously published except using a sheep anti-mouse Ig HRP conjugated antibody for detection (Amersham, Piscataway, NJ)[40].

ELISPOT

The protocol has been previously described in more detail[40]. Plates were coated with 5 μg/ml anti-interferon-γ (IFN-γ) or anti-interleukin-4 overnight (BD Pharmigen). The next day 1 x 10^5 cells were incubated with media containing DMSO, H peptide pool (20mers with 11aa overlap) at 5 μg/ml, MV lysate diluted 1:100, or 5 μg/ml of ConA for 36 hours. The plates were then washed and incubated with biotinylated rat anti-mouse IFN-γ or rat anti-mouse IL-4 antibodies at 2 μg/ml (BD Pharmigen).

Microarrays

Total RNA was isolated from cells using the RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's recommended protocol. Quality assessment was determined by RNA Nano LabChip analysis on an Agilent Bioanalyzer 2100 (Agilent, Palo Alto, CA).

Processing of templates for GeneChip Analysis was in accordance with methods described in the Affymetrix GeneChip Expression Analysis Technical Manual, revision five, utilizing reagents from the GeneChip Expression 3' Amplification One-Cycle Target labeling kit (Affymetrix, Santa Clara, CA). For each sample, double stranded cDNA was synthesized from 1 μg of total RNA. Following column purification, an *in vitro* transcription reaction was performed. This reaction incorporated biotinylated ribonucleotides to produce labeled antisense cRNA targets for hybridization. Purification of biotinylated cRNAs was performed with the GeneChip Sample Cleanup Module (Affymetrix). Fifteen micrograms of cRNA was fragmented by metal-induced hydrolysis in fragmentation buffer (250mM Tris acetate pH 8.1, 150 mM MgOAc, 500mM KOAc) at 94^o C for 35 minutes. Hybridization cocktails were prepared as recommended for

arrays of "Standard" format including incubation at 94°C for 5 minutes and 45°C for 5 minutes, and centrifugation at maximum speed for 5 minutes prior to pipetting into the GeneChips (Affymetrix Mouse Genome 430 2.0). Hybridization was performed at 45°C for 16 hours at 60 rpm in the Affymetrix rotisserie hybridization oven. The signal amplification protocol for washing and staining of eukaryotic targets was performed in an automated fluidics station (Affymetrix FS450) as described in the Affymetrix Technical Manual, Revision Five. The arrays were then transferred to the GCS3000 laser scanner and scanned at an emission wavelength of 570 nm at 2.5 mm resolution (Affymetrix). Intensity of hybridization for each probe pair was computed by GCOS 1.2 software (Affymetrix).

The CEL file images were viewed and affyPLM was used to determine outliers in quality control. Data was normalized using R and Robust Multi-Array Analysis (RMA) v1.5.8 and significance was determined using Significance Analysis of Microarrays (SAM) v2.21[41, 42, 43]. Day 0 controls and saline controls at each timepoint were combined if there were no significantly regulated genes found between the samples. Pairwise comparisons were then made between the control and vaccine samples at each timepoint. Significant gene lists were annotated using Onto-Express[44].

Results

Serum Antibodies and ELISPOTs

Serum antibody levels were measured against whole MV lysate. FIMV induced a more rapid response than VCR-H, but final titers were similar between the two vaccines, while saline alone did not induce antibody production (Figure 4.1). Titers were still

increasing at day 42. The quality of this antibody is not the same between the vaccines though, as the FIMV induces an antibody with lower avidity (J. Bergen, unpublished data).

Spleen and lymph node cells IFN-γ production was rapidly induced to the H peptides and whole MV lysate by VCR-H, with the peak occurring at day 7 post-vaccination. The MV-specific cell number was maintained in the spleen through day 42 while it slowly declined in the lymph node (Figure 4.2bc-4.3bc). Many more cells responded to the H peptides than to whole MV lysate, which may reflect the dose of antigen that was used or the need for antigen processing. The FIMV did induce some IFN-γ producing cells, but to a lower magnitude and it was transient. There was a low level of non-specific IFN-γ production in mice that received the VCR-H (Figures 4.2a and 4.3a). IL-4 production was not different between the two vaccine groups and saline controls (Supplemental Figures 4.1-4.2).

To determine which cells were producing the IFN-γ, CD4+ T cells or CD8+ T cells were depleted using magnetic beads. In general, depletion of CD4+ T cells decreased the response, while depletion of CD8+ T cells increased the response against the H peptide (Figure 4.4a). However, neither eliminated the response, suggesting that both cell types contributed to IFN-γ production. It is also possible that another cell type contributes to IFN-γ secretion. The IFN-γ response to stimulation with whole virus lysate was primarily due to CD4+ T cells because CD4 depletion significantly reduced the number of spots and CD8+ T cell depletion increased the number of spots (Figure 4.4b).

Microarray Analysis

To understand the molecular differences in response to the two vaccines, microarray analysis was conducted on enriched CD4+ T cell, CD8+ T cell and CD19+ B cell populations from the spleen on days 0, 4, 7 and 28 for CD8+ T cells and days 0 and 4 for CD19+ B cells. A summary of the complete results is shown in Table 4.1. There were only 2 significantly regulated genes in the CD4+ T cell samples. On day 4, FIMV significantly induced 206 genes in the CD19+ B cells group while VCR-H did not induce any genes in B cells. VCR-H induced 388 genes and down regulated 561 genes in CD8+ T cells on day 4, but FIMV did not show any changes until day 28 when it induced 60 genes and down regulated 142. These significantly regulated genes were then grouped into gene ontology biological processes. The complete tables of significantly regulated genes and the genes found in each significant group are shown in Supplemental Tables 4.1-4.10.

CD19+ B cells FIMV Day 4

There were six groups significantly up regulated in these samples for B cells: regulation of transcription, DNA-dependent, transcription, nuclear mRNA splicing, via spliceosome, mRNA processing, protein amino acid phosphorylation and cell cycle (Table 4.2). The first four groups are all involved in transcription. The genes found in these groups are predominantly transcription factors including foxp1, early B cell factor 1 and transacting transcription factors 1 and 3. The fifth group, protein amino acid phosphorylation, was composed entirely of kinases. The final group, cell cycle, had a mixture of genes. The only significant cellular component was the nucleus with 45 genes significantly regulated.

CD8+ T cells VCR-H Day 4

There were 388 up regulated and 561 down regulated genes induced by the VCR-H vaccine in spleen CD8+ T cells at day 4 (Table 4.1). By day 7 there was only 1 significantly up regulated gene and none at day 28. The up regulated genes at day 4 did not fall into any significantly regulated gene groups.

There were a large number of significantly down regulated groups of genes (Table 4.3). The top groups were involved in transcription and amino acid phosphorylation. The transcription-related groups included a number of splicing factors, STAT1, STAT3, STAT5a, transcription and binding factors. The amino acid phosphorylation group was mainly composed of kinases. After transcription and phosphorylation, the next groups were regulation of translation, Wnt receptor signaling pathway, cell cycle, intracellular signaling cascade, RNA processing, etc. Many of the genes in these groups overlap with the transcription and phosphorylation groups.

Groups with unique genes included the cell surface receptor linked signal transduction group where there were three cytokine receptors that were down regulated: IL-4R, IL-6R and IL-17R. The protein amino acid dephosphorylation group was mostly phosphatases.

The significantly regulated cellular compartments included the nucleus, cytoplasm, spliceosome complex, intracellular, cytosol and ribonucleoprotein complex, which supports the finding that transcription is down regulated at this timepoint (Table 4.4).

CD8+ T cells FIMV Day 28

The only up regulated group of genes in day 28 CD8+ T cells from the spleens of FIMV vaccinated mice was development (Table 4.5). It included sonic hedgehog and

two snail homolog genes (Supplemental Table 4.9). Interestingly, the extracellular space was a significantly up regulated cellular component, although the genes were not generally immune related genes, except for one member of the complement component cascade.

The main gene groups identified for the down regulated genes were involved in transcription and included Sp1 and STAT3. About 50% of the genes down regulated in this group were also down regulated on day 4 following VCR-H vaccination. The only significantly down regulated cellular component was the nucleus.

Discussion

In this study, we compared the VCR-H vaccine to the FIMV developed in the 1960's. Both vaccines induced a similar antibody titer against whole MV lysate, although antibodies induced by the FIMV were of lower avidity (J. Bergen, unpublished data). There was a profound difference in the induction of IFN-γ producing cells. The VCR-H vaccine induced a robust response, while the FIMV induced a weaker, more transient response in both the spleen and the lymph node. Depletion studies showed that the VCR-H IFN-γ response to the whole virus antigen was predominantly from CD4+ T cells, but the response to H peptides was both CD4+ and CD8+ T cells or potentially other cell types.

Microarray analysis was used to compare the molecular changes in CD19+ B cells, CD4+ T cells and CD8+ T cells following vaccination. There were only 2 significantly regulated genes in the CD4+ T cell sample, possibly due to insufficient enrichment of antigen-specific CD4+ T cells. On day 4 post-vaccination, CD19+ B cells

from mice inoculated with FIMV up regulated 206 genes involved in transcription, phosphorylation and the cell cycle. Many of the up regulated genes, such as early B cell factor (EBF), special AT-rich sequence binding protein-1 (SATB1), BTB and CNC homology 2 (Bach2), Creb binding protein (CBP), Sp1, Sp3 and forkhead box P1 (Foxp1) have been characterized in B cells.

EBF is specific for B cells and is required for immunoglobulin expression and control of differentiation[45, 46, 47]. SATB1, which can act as either a repressor or activator, is a docking site for chromatin-remodeling enzymes and is known to be involved in B cell tolerance[48, 49, 50]. The B cell-specific, transcriptional repressor Bach2 is critical for class switch recombination and somatic hypermutation[51, 52, 53]. It is expressed in proliferating germinal center B cells, but is shut-down as B cells differentiate into plasma cells[54]. MHC class II gene transcription is increased by CBP and, when overexpressed, CBP strongly stimulates cell proliferation[55, 56]. CBP is known to interact with EBF, HIF-1α and Sp3. Also, Sp1 regulatory elements are found upstream of the transcription start site[56, 57]. HIF-1α is involved in the maturation of B cells and Foxp1 is expressed in germinal center B cells[58, 59]. Sp1 and Sp3 are general transcriptional activators that often compete for the same binding sites[60]. Overall, these genes suggest an activated, proliferating germinal center B cell response.

There were 388 up regulated and 561 down regulated genes on day four post-vaccination in the CD8+ T cells of VCR-H inoculated mice. The up regulated genes did not fall into any significantly up regulated groups, possibly because many have not been well-characterized. The down regulated genes were largely involved in transcription and phosphorylation. There are some well-studied genes including B cell

leukemia/lymphoma 11B (Bcl11b), GATA binding protein 3 (GATA-3), lymphoid enhancer binding protein (LEF1), Sin3a and signal transducers and activators of transcription 1, 3, and 5a (Stat) genes. Bcl11b is a key regulator of T cell differentiation and survival[61]. GATA3 binds to TCRα gene enhancer and is required for the expression of TCR α, β and δ and CD8α[62, 63]. Forced GATA-3 expression inhibits CD8 maturation[64]. Wnt signaling includes LEF1, which is down regulated following antigen encounter[65, 66]. Sin3a functions as a platform for histone deacetylase enzymes and other chromatin-modifying enzymes[67]. T cell numbers and proliferative potential are reduced in Sin3a knockout mice[67]. T cell subset expansion depends on low Stat1 levels and hence, proliferating, antigen-specific T cells have low Stat1 expression[68]. Stat5 is required for T cell homeostasis and knockout animals have fewer CD8 T cells[69, 70]. Activation of both Stat3 and Stat5 promotes proliferation and survival, so the down regulation may mean a decreasing cell population[71]. This gene expression signature suggests a dynamic CD8+ T cell population that is probably maturing and expanding, but also experiencing increased cell death.

The FIMV did not induce significant changes in CD8+ T cells until 28 days post-vaccination when 60 genes were up regulated and 142 were down regulated. Development was the only up regulated group while transcription was down regulated. The characterized, down regulated genes included Sp1, cellular nucleic acid binding protein 1 (CNBP) and nemo-like kinase (NLK). Sp1 may regulate CNBP, which stimulates cell proliferation[56]. NLK works to degrade LEF/TCF complex, which suppresses Wnt signaling[72, 73]. There was significant overlap between the VCR-H day 4 results and the FIMV day 28 results, with about 50% of the day 28 genes also down

regulated on day 4. The reason for the delayed changes in CD8+ T cells following FIMV vaccination is unclear, but the overlapping gene expression profiles show a degree of similarity in the CD8+ T cell immune response. Further research is needed to understand the extent of the overlap and the functional consequences of the differences between the expression profiles.

The stark contrast between the immune responses to the VEE and FIMV vaccines provides an excellent system to study the necessary components for effective vaccination. Microarray analysis also provides a powerful method to analyze the molecular differences induced by the two vaccines in immune cell subsets of the mouse. By further exploring this system and the contrasting immune responses, scientists can learn important principles to guide rationale vaccine design.

Acknowledgements

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Table 4.1. Microarray analysis results for spleen CD4+ T cells, CD8+ T cells and CD19+ B cells.

		Day 4	**Day 7**	**Day 28**
CD4+ T cells	Saline	0	0	0
	VCR-H	0	0	0
	FIMV	0	2 up	0
CD8+ T cells	Saline	0	0	0
	VCR-H	388 up 561 down	1 up	0
	FIMV	0	0	60 up 142 down
CD19+ B cells	Saline	0	ND	
	VCR-H	0		
	FIMV	206 up		

ND= Not Determined

Table 4.2. Significantly up regulated biological processes in CD19+ B cells on day 4 from mice inoculated with FIMV.

GO ID	Function Name	Input Total	Corrected P-Value
6355	Regulation of transcription, DNA-dependent	28	2.32E-04
6350	Transcription	21	0.001
398	Nuclear mRNA splicing, via spliceosome	8	0.002
6397	mRNA processing	8	0.004
6468	Protein amino acid phosphorylation	9	0.016
7049	Cell cycle	5	0.037

Table 4.3. Significantly down regulated biological processes in CD8+ T cells on day 4 from mice inoculated with VCR-H.

GO ID	Function Name	Input Total	Corrected P-Value
6397	mRNA processing	31	0
6350	Transcription	65	1.69E-10
6355	Regulation of transcription, DNA-dependent	80	1.85E-10
398	Nuclear mRNA splicing, via spliceosome	24	1.26E-08
6468	Protein amino acid phosphorylation	30	8.55E-06
45944	Positive regulation of transcription from RNA polymerase II promoter	11	6.62E-05
6366	Transcription from RNA polymerase II promoter	6	0.002
6445	Regulation of translation	5	0.003
16055	Wnt receptor signaling pathway	7	0.004
7049	Cell cycle	15	0.004
7242	Intracellular signaling cascade	18	0.004
6396	RNA processing	5	0.005
16481	Negative regulation of transcription	5	0.005
6357	Regulation of transcription from RNA polymerase II promoter	6	0.010
6915	Apoptosis	12	0.012
9887	Organogenesis	8	0.013
7166	Cell surface receptor linked signal transduction	5	0.014
7283	Spermatogenesis	6	0.015
6512	Ubiquitin cycle	13	0.016
50875	Cellular physiological process	6	0.027
6974	Response to DNA damage stimulus	7	0.032
7264	Small GTPase mediated signal transduction	8	0.034
6260	DNA replication	5	0.034
6470	Protein amino acid dephosphorylation	6	0.035
4	Biological process unknown	25	0.036
6605	Protein targeting	6	0.043
910	Cytokinesis	7	0.046

Table 4.4. Significantly down regulated cellular components in CD8+ T cells on day 4 from mice inoculated with VCR-H.

GO ID	Cellular Component	Input Total	Corrected P-Value
5634	Nucleus	163	1.93E-09
5737	Cytoplasm	46	3.92E-06
5681	Spliceosome complex	10	5.90E-05
5622	Intracellular	25	0.019
5829	Cytosol	11	0.021
30529	Ribonucleoprotein complex	12	0.043

Table 4.5. Significantly regulated biological processes in spleen CD8+ T cells from mice on day 28 after inoculation with FIMV.

GO ID	Function Name	Input Total	Corrected P-Value
	Up regulated		
7275	Development	6	5.52E-04
	Down regulated		
398	Nuclear mRNA splicing, via spliceosome	12	3.34E-08
6397	mRNA processing	13	3.52E-08
6350	Transcription	12	0.011
4	Biological process unknown	8	0.022
6355	Regulation of transcription, DNA-dependent	13	0.027

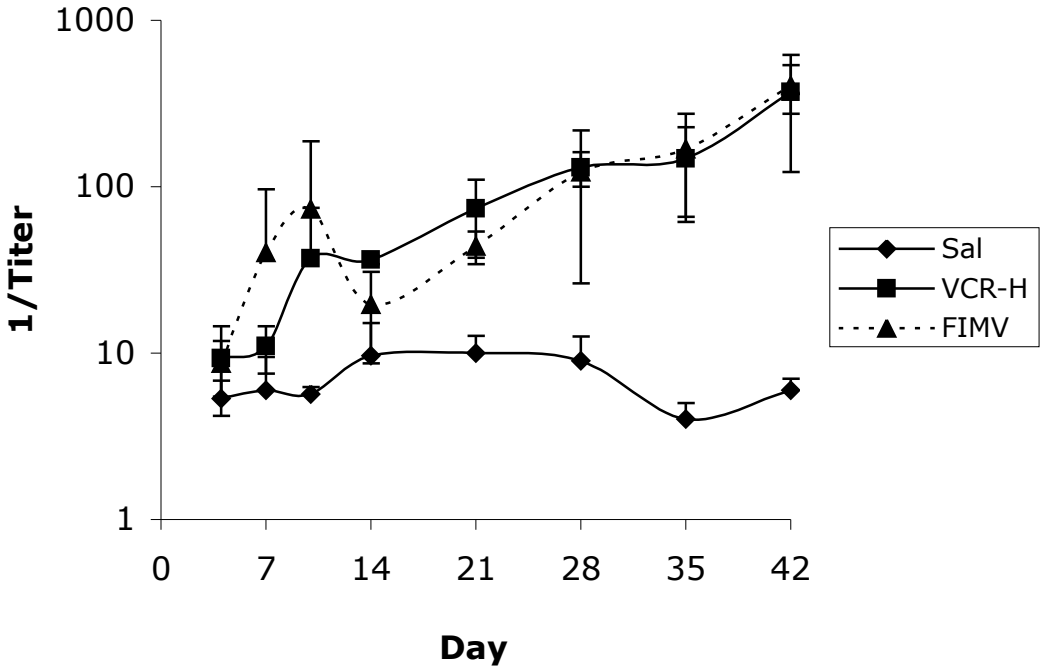

Figure 4.1 Serum antibody titers against whole measles virus lysate. BALB/c mice were inoculated with saline, VCR-H or FIMV vaccines on day 0 and serum was collected on the indicated days.

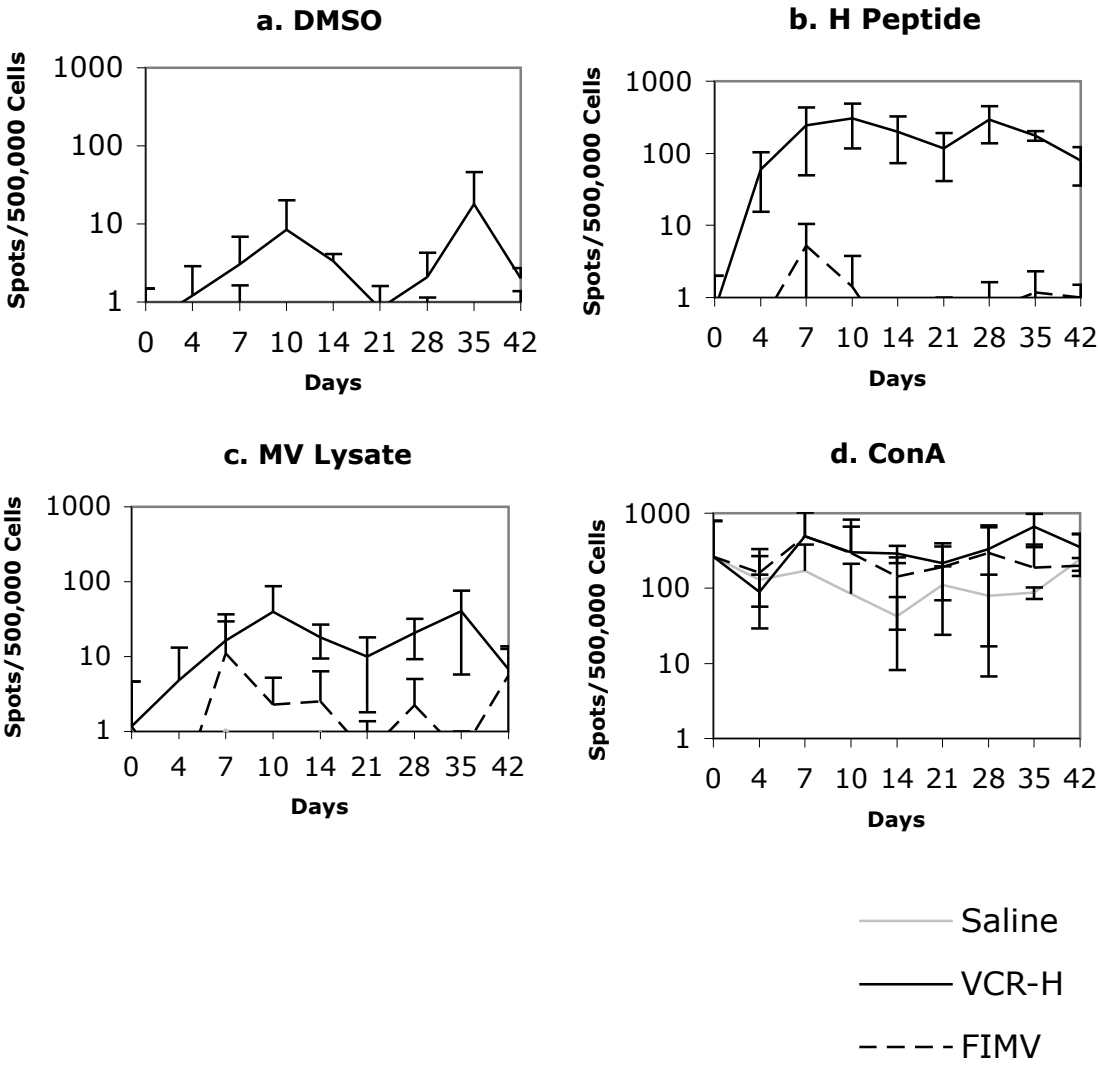

Figure 4.2 IFN-γ secreting cells in the spleens of vaccinated mice. BALB/c mice were inoculated with saline, VCR-H or FIMV vaccines and spleens were removed on the indicated days. Isolated splenocytes were stimulated with (a) dimethyl sulfoxide (DMSO), (b) MV H peptide, (c) whole MV lysate or (d) concanavalin A (ConA) for 36 hours and IFN-γ secreting cells were detected by ELISPOT.

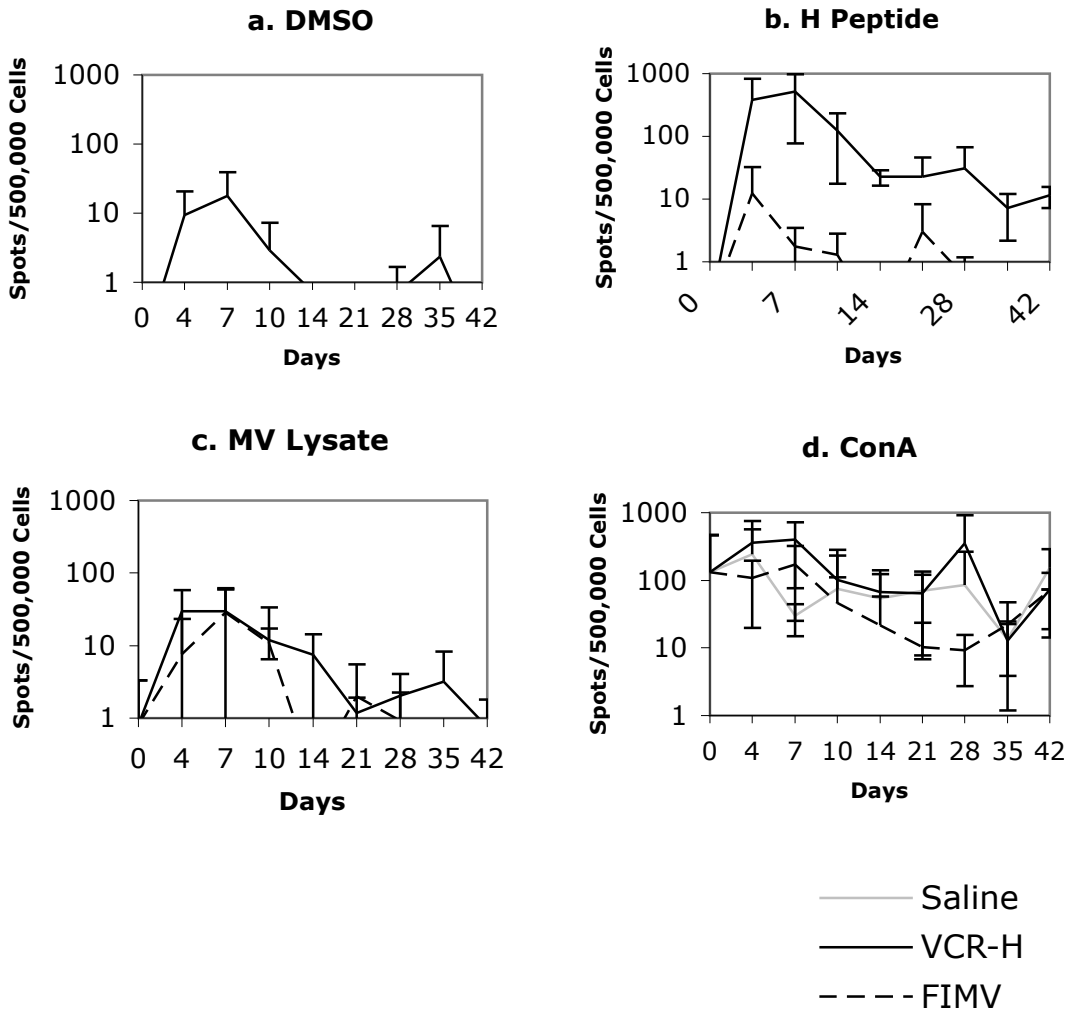

Figure 4.3 IFN-γ secreting cells in the lymph nodes of vaccinated mice. BALB/c mice were inoculated with saline, VCR-H or FIMV vaccines and lymph nodes were removed on the indicated days. Isolated lymph nodes were stimulated with (a) dimethyl sulfoxide (DMSO), (b) MV H peptide, (c) whole MV lysate or (d) concanavalin A (ConA) for 36 hours and IFN-γ secreting cells were detected by ELISPOT.

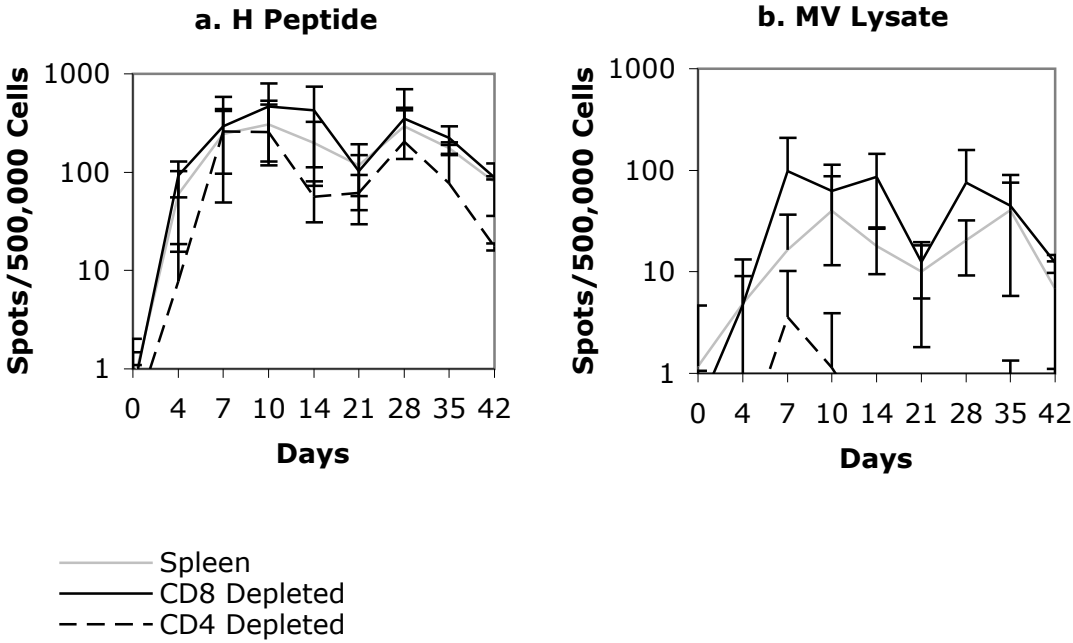

Figure 4.4 IFN-γ secreting cells in the spleens of vaccinated mice depleted of CD4+ or CD8+ T cells. BALB/c mice were inoculated with saline, VCR-H or FIMV vaccines and spleens were removed on the indicated days. CD4+ or CD8+ cells were removed by magnetic bead separation. Enriched cells were stimulated with (a) MV H peptide or (b) whole MV lysate for 36 hours and IFN-γ secreting cells were detected by ELISPOT.

Summary

By integrating biology, statistics and computation, microarray analysis has emerged as one of the most powerful technologies in biological research. To harness the power of this technology, we developed an *in vitro* infection model where dendritic cells were experimentally infected and analyzed over the course of 24 hours. These results were compared to other pathogen-host cell infection models to understand the cellular response to pathogens. This approach identified PKR as a unique gene in MV infection, where it was not induced, while the other pathogens did induce this gene. The interferons also showed an interesting pattern as all of the IFN-α genes were induced in measles, but only a few were induced in influenza, the only other virus that was compared. None of the other pathogens induced IFN-α genes. The metallothioneins were a novel group of genes that were widely induced by all pathogens in the DC-infection systems. HIV infection of PBMCs and HCMV infection of fibroblasts did not induce any metallothioneins. This *in vitro* infection model showed that microarrays could provide novel insights into pathogen-host cell relationships and that results could be compared between laboratories.

Next, we examined acute disease in Zambian children admitted to the hospital for measles infection. There were several genes found to be up regulated at discharge and follow up including chemokines, such as CXCL2, CCL4 and IL-8, and the cytokines IL-1β and TNF-α. The down regulated genes were mainly involved in transcription and signal transduction. There are difficulties interpreting the results from the human samples, because the cell composition of the PBMC compartment was changing over time. The largest changes in cellular composition were at entry and there were no

significantly regulated genes at that timepoint, suggesting the results reflect gene expression changes.

Finally, two vaccines were compared in mice. Following vaccination with either saline, VCR-H or FIMV, CD4+ T cells, CD8+ T cells and B cells were isolated at several timepoints. The antibody titer was similar between the two vaccines, although other unpublished work in the lab has shown that FIMV induces a lower avidity antibody. The cellular immune responses were very different as VCR-H induced a robust IFN-γ response in both the spleen and lymph nodes while FIMV induced a weaker and more transient response. Microarray analysis identified a number of up regulated transcription factors and genes involved in signal transduction in B cells on day 4 following FIMV vaccination. The overall transcriptional response indicated an activated and proliferating germinal-center B cell population. A number of transcription factors were down regulated on day 4 following VCR-H vaccination and much later, at day 28, for FIMV. There was approximately 50% overlap between these two sets of down regulated genes and it suggested a mixed response with significant cellular expansion and increased cell death.

These results show the applicability of microarray technology to a wide array of biological research. The power to generate hypotheses will expand the influence of microarray technology throughout biology as the raw materials become cheaper and the analysis becomes more standardized. Undoubtedly, the ability of microarrays to characterize a tissue or cell sample through the expression of thousands of genes will transform biological research and medical science, profoundly improving the human condition.

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Employment

1999 – Present Johns Hopkins University Baltimore, MD

PhD Candidate

Project: Measles virus infection of dendritic cells *in vitro*, gene expression during natural measles *in vivo*, and experimental vaccine research.

- I have focused on using microarray technology to investigate measles virus interactions with the host immune system. First, I looked at measles virus-dendritic cell interactions *in vitro.* Second, I have examined the gene expression changes that occur following acute infection in peripheral blood mononuclear cells. Finally, I am analyzing two measles virus vaccines that show different immune activation profiles to understand the transcriptional differences between the vaccines.

2001-2003

Teaching Assistant

Class: Principles of Immunology I and II, Topics in Immunology I and II

- Responsibilities included holding a weekly discussion session, lecturing and grading exams.

1998-1999 University of Minnesota St. Paul, MN

Junior Scientist

Project: Characterization of porcine interleukin-18.

- Interleukin-18 is an important cytokine involved in the

inflammatory response to pathogens. The project involved cloning the porcine interleukin-18 gene, expressing the protein in a bacterial expression system and characterizing its tissue distribution.

1996-1998
Undergraduate Research Assistant
Project: Opioid receptor expression on immune cells.

- The aim of the project was to determine if the μ, κ and δ opiate receptors were present on immune cells. This required developing nested PCR reactions and Southern blots for the receptors followed by screening mRNA isolated from B cells, T cells, and monocytes.

Education

1999-Present Johns Hopkins University Baltimore, MD
PhD Candidate

1993 – 1998 University of Minnesota Minneapolis, MN
Bachelor of Science, Magna Cum Laude
Major: Biology and Philosophy

Publications

Zilliox, M.J., Parmigiani, G., and Griffin, D.E. 2006. Gene expression patterns in dendritic cells infected with measles virus compared with other pathogens. *PNAS* 103:3363-3368.

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